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1615

PATENT
Attorney Docket No. **CONLINCO-03586**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Asgeir Saebo *et al.*		
Serial No.:	09/132,593	Group No.:	1615
Filed:	08/11/1998	Examiner:	Wang
Entitled:	**CONJUGATED LINOLEIC ACID ALKYL ESTERS IN FEED STUFFS AND FOOD**		

TRANSMITTAL OF APPELLANTS' REPLY BRIEF

APPEAL NO.:

Mail Stop Appeal Brief - Patents
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Dated: December 6, 2004 By: _____
Mary Ellen Waite

Sir:

Submitted herewith in triplicate is Appellants' Reply Brief to the Examiner's Answer mailed November 4, 2004. It is not believed that any fees are necessary for this reply. However, if any fees are necessary, the Examiner is hereby authorized to charge Deposit Account No. 08-1290 the fee associated with this Reply Brief and any other fees associated with this communication. Please reference Attorney Docket No.: CONLINCO-03586 when charging the Attorney Deposit Account. A request for oral examination is being filed concurrently herewith.

Dated: December 6, 2004

_____
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Dated: December 6, 2004 By: Mary Ellen Waite

Sir:

This Brief is in reply to the Examiner's Answer mailed November 04, 2004.

It is not believed that any fees are necessary for this reply. However, if any fees are necessary, the Examiner is hereby authorized to charge Deposit Account No. 08-1290 the fee associated with this Reply Brief and any other fees associated with this communication. Please reference Attorney Docket No.: CONLINCO-03586 when charging the Attorney Deposit Account. A request for oral examination is being filed concurrently herewith.

This Brief is transmitted in triplicate. [37 C.F.R. § 1.192(a)].

ARGUMENT

The Office's acceptance of the statements of the real party in interest, status of claims, status of amendments after final, summary of invention, and issues, and grouping of the claims is appreciated.

Below, Appellants specifically address the following issues from the initial Appeal Brief:

Issue 1 – Whether Claims 1-6 are not obvious over the combination of Cook et al. (U.S. Patent No. 5,554,646), Cain et al. (WO97/18320), Chin et al. (Journal of Food Composition and Analysis, 1992, Vol. 5, pages 185-197), and Baltes et al. (U.S. Patent No. 3,162,658).

A. Claims 1-6 and 8 Are Not Obvious Over The Combination of Cook et al., Cain et al., Chin et al., and Baltes et al.

Claims 1-6 and 8 stand rejected under 35 U.S.C. §103 as allegedly being obvious over the combination of Cook et al., Cain et al., Chin et al., and Baltes et al. The Office has failed to establish a *prima facie* case of obviousness because the references, alone or in combination, fail to teach each element of the claimed compositions. In particular, the combined references do not teach alkyl esters comprising less than about two percent trans,trans; 8,10 and 11,13 octadecadienoic acid isomers.

Regarding Cook et al., the Office alleged the following:

> Cook et al. do not teach expressly the conjugated linoleic acid form further comprising the regio isomers 8,10- and an 11,13- octadecadienoic acid derivative...However, since the preferred amounts of the regio isomers 8,10- and an [sic] 11,13-octadecadienoic acid derivative in the claimed invention are limited to less than 2 percent, this amount includes zero percent of the regio isomers as disclosed by Cook. Thus, Cook' teachings meet this limitation.

Paper 41, page 3.

Regarding Cain et al., the Office alleged the following:

> Cain et al. further teaches a CLA composition made from sunflower oil for food additive contains 48.9% of c9, t11, 51.1% of t10, c12 linoleic acid or their esters. See, particularly, examples 6 at page 16, and example 18 at page 36, and claim 7.

Paper, pages 3-4.

In response to the rejections over Cook et al. and Cain et al., Applicants submitted Declarations from Asgeir Sæbo (copies attached hereto as Appendix B for the Office's convenience) that establishes that the compositions of Cook et al. and the compositions of Cain et al. cannot produce alkyl esters comprising less than about two percent trans,trans; 8,10 and 11,13 octadecadienoic acid isomers. In response to the Sæbo Declarations, the Office stated the following:

> The declaration fails to establish the fact that the conjugated linoleic acid disclosed by Cook or Cain as recited in the prior office action containing more than 2% of the isomers identified in the claim herein. Particularly, applicant generated data, proffered to obviate prior art teachings, lacks the probative force accorded data generated by independent, disinterested parties. It is well settled patent law 'that it is not a difficult matter to carry out a process in such a fashion that it will not be successful and, therefore, the failures of experimenters who have no interest in succeeding should not be accorded great weight' In re Michalek, 74 USPQ 108, at 109 citing Bullard Company et al v. Coe, 147 F.2d 568, 64 USPQ 359.

Paper 34, pages 2-3. Nearly identical arguments are presented in the Answer to the Appellant's Appeal Brief at pages 3-5. For the following reasons, Applicants respectfully submit that the Office's reliance on this standard is unfounded.

First, contrary to the Office's assertion and as discussed in more detail below, the Sæbo Declarations establish that compositions made by the methods of Cook et al. and Cain et al. contain more the 2% of the identified isomers.

Second, Applicants respectfully submit that the Office's "well-settled" case law is not settled at all, and, in fact, is not in accordance with proper PTO practice. The decision relied on by the Office is more than 50 years old. While this case has not been directly overruled, it is in

conflict with current case law and PTO practice. In particular, the Office's failure to accord the proper weight to the Sæbo Declarations does not conform with proper patent practice according the Manual of Patent Examining Procedure (MPEP). The Office must respond to all of the arguments and evidence presented by Applicants. The MPEP states that:

> **Office personnel should consider all rebuttal arguments and evidence presented by applicants**. . . . *In re Beattie*, 974 F.2d 1309, 1313, 24 USPQ2d 1040, 1042-43 (Fed. Cir. 1992). . . . **Office personnel should avoid giving evidence no weight**, except in rare circumstances. *Id. See also In re Alton*, 76 F.3d 1168, 1174-75, 37 USPQ2d 1578, 1582-83 (Fed. Cir. 1996).
>
> * * *
>
> A determination under 35 U.S.C. 103 should rest on **all the evidence** and should not be influenced by any earlier conclusion. *See, e.g., Piasecki*, 745 F.2d at 1472-73, 223 USPQ at 788; *In re Eli Lilly & Co.*, 902 F.2d 943, 945, 14 USPQ2d 1741, 1743 (Fed. Cir. 1990). Thus, once the applicant has presented rebuttal evidence, Office personnel should **reconsider** any initial obviousness determination in view of the entire record. *See, e.g., Piasecki*, 745 F.2d at 1472, 223 USPQ at 788; *Eli Lilly*, 902 F.2d at 945, 14 USPQ2d at 1743.[1]

Additionally, the Courts have held as follows:

> When *prima facie* obviousness is established and evidence is submitted in rebuttal, the decision-maker must start over . . . . An earlier decision should not . . . be considered as set in concrete, and applicant's rebuttal evidence then be evaluated only its knockdown ability. Analytical fixation on an earlier decision can tend to provide the decision with an undeservedly broadened umbrella effect. *Prima facie* obviousness is a legal conclusion, not a fact. Facts established by rebuttal evidence must be evaluated along with the facts on which the earlier conclusion was reached, not against the conclusion itself. Though the tribunal must begin anew, a final finding of obviousness may of course be reached, but such finding will rest upon evaluation of all facts in evidence, uninfluenced by any earlier conclusion reached . . . upon a different record.[2]

Furthermore:

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[1] MPEP §§2144.08; emphasis added.

[2] *In re Rinehart*, 531 F.2d 1048, 1052, 189 USPQ 143, 147 (CCPA 1976).

> If a *prima facie* case is made in the first instance, and if the applicant comes forward with a reasonable rebuttal, **whether buttressed by experiment**, prior art references, or argument, the entire merits of the matter are to be reweighed. (Emphasis added)[3]

Accordingly, even if the Office had established a *prima facie* case of obviousness (and Applicants contend that the Office did not), the Office must respond to the information presented in the Declaration. The above directions of the court and the PTO state that the evidence **must be considered**. These directions do not categorize evidence according to whether it is developed by interested or disinterested parties and do not state that evidence developed by the inventor **may be ignored. The directions specifically state that experimental evidence, such as that contained in the Declaration, must be considered.** Indeed, the Office must start over and reconsider the entire obviousness analysis.

Any distinction between giving evidence "no weight" or "great weight," based upon the teaching of *In re Michalek*, does not relieve an Office from addressing the cited holdings. Indeed, the Office must address the cited holdings of *In re Rinehart*, 531 F.2d 1048, 1052, 189 USPQ 143, 147 (CCPA 1976) and *In re Hedges*, 783 F.2d 1038, 1039, 228 USPQ 685, 686 (Fed. Cir. 1986).

In the Answer to Appellant's Appeal Brief, at page 7, the Office asserts that *In re Michalek* does not conflict with current PTO practice. In particular, the Office alleges:

> all the arguments presented by the applicants, including the evidence submitted with the declaration, have been fully considered, but are found not persuasive. The Office has not ignored appellant's evidences. All the facts, including appellant's evidences have been fully evaluated. When applicants' evidence is not consistent with the teaching from prior art, *In re Michalek* is properly applied. The Office fails to see any conflict between the *In re Michalek* decision and current case law or current PTO practice" (Answer to Appellant's Appeal Brief, page 7).

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[3] *In re Hedges*, 783 F.2d 1038, 1039, 228 USPQ 685, 686 (Fed. Cir. 1986).

The Office is not addressing the fact that experimental evidence, such as that provided in the Declaration, must be considered and evaluated. In particular, per *In re Hedges* and *In re Rinehart*, the Office must **respond** to the information presented in the Declarations in a manner indicating that the evidence was considered. The Office's conclusory statements do not indicate that evidence provided in the Declarations was properly considered and evaluated.

Indeed, in the present case, there was no reweighing of the merits by the Office. Instead of actually analyzing the Declarations and the factual, experimental data contained within them, the Office has summarily dismissed the data with no analysis because it was generated by the Applicants. Applicants submit that the results would be the same no matter who conducts the experiments. **The Office has failed to substantively address the data in the Declarations and offer any analysis of why the data is flawed.** As explained in detail below, *In re Michalek* is both legally and factually distinguishable.

1. ***In re Michalek* is Legally Distinguishable**

In their Appeal Brief, the Applicants established that *In re Michalek* is a case that is limited to its particular facts. In the Answer to Appellant's Appeal Brief, the Office does not address the fact that *In re Michalek* is limited to its particular facts. *In re Michalek* involved claims to a thermoplastic resin comprising polymers or copolymers of one or more nuclear-substituted dichlorostyrenes. *In re Michalek*, 34 C.C.P.A. 1124; 162 F.2d 229; 74 U.S.P.Q. 107 (1947). The court stated the following with respect to the affidavits submitted in support of patentability:

> Several affidavits to sustain appellant's contention were filed. In substance it is stated therein that the processes of the Dreisbach patents do not produce monomeric dichlorostyrene sufficiently pure to meet the density and index of refraction recited in the claims or to polymerize, although it is not said that the processes of the patents are

> incapable of producing the nuclear-substituted dichlorostyrenes mentioned therein. The board considered the affidavits insufficient to overcome the definite naming in those patents of dichlorostyrene and the indication that it is polymerizable. In this connection the solicitor in his brief cites *In re Von Bramer, 29 C.C.P.A. 1018, 127 F.2d 149, 53 U.S.P.Q. 345.*
>
> With respect to the experiments described in the affidavits it must be said that in a patent it is to be presumed that a process, if used by one skilled in the art, will produce the product alleged by the patentee and such presumption is not overcome by a mere showing that it is possible to operate within the disclosure without obtaining the alleged product. Skilled workers would as a matter of course, in our opinion, if they do not immediately obtain desired results, make certain experiments and adaptations and we agree with the argument of the solicitor that it is not a difficult matter to carry out a process in such fashion that it will not be successful and, therefore, the failures of experimenters who have no interest in succeeding should not be accorded great weight, citing *Bullard Company et al. v. Coe, 79 U.S. App. D.C. 369, 147 F.2d 568, 64 U.S.P.Q. 359.* Possibly more extensive experiments than were made by the affiants herein might have produced a different result.

It is clear from this discussion that the holding of the court was specific to the facts presented to it, and especially to the nature of the data contained in the affidavits. The court emphasizes this by stating "more extensive experiments" might have "produced a different result." Thus, *In re Michalek* **does not stand** for the proposition that all reproductions of prior art data by an inventor should be ignored simply because the inventor has no reason to be successful. Instead, the court in *In re Michalek* evaluated the data and found it insufficient. The Office has failed to do this in the instant case. As indicated above, the Office has provided no reasoning as to why the data submitted is insufficient or incorrect. As described in more detail below, this is reversible error.

2. *In re Michalek* Is Factually Distinguishable

In re Michalek is also factually distinguishable. The Office has attempted to ignore the evidence provided by Applicants by relying on *In re Michalek* and characterizing the evidence as

a failure. To the contrary, the evidence presented by the Applicants is not a failure to repeat the results of Cook et al. and Cain et al. Both Cook et al. and Cain et al. are **silent** as to the presence of the 8,10 and 11,13 isomers. The only way the Applicants results could be considered to be a failure is if Cook et al. and Cain et al. affirmatively stated that the isomers were not present and then Applicants failed not to produce the isomers. This is not the present situation. Applicant's results supplement the teachings of Cook et al. and Cain et al., and do not contradict them.

In the Answer to Appellant's Appeal Brief at page 8, the Office admits, "[i]t is noted that the Office could not give more detailed analysis to the data presented in the declaration since the declaration merely states that appellant has repeated the experiments in Cain et al. It is assumed that appellant just simply duplicated an experiment disclosed by Cain et al. In the instant case, like in re Michalek, more extensive experiments might have produced a different result."

The Office is misinterpreting and misapplying *In re Michalek*. First, the Declarations noted in *In re Michalek* was deemed insufficient because it failed to completely address the claimed material. Here, the Declarations provided by the Applicants establish that the methods used by Cain et al. and Cook et al. **could not** produce compositions comprising *less than* 2% trans,trans 8,10 and 11,13 octadecadienoic acid isomers. Second, the Office admits that a proper evaluation of the evidence contained within the Declarations was not conducted because the Declaration merely states that it had conducted repeat experiments. *In re Michalek* described a scenario where a Declaration based upon evidence gathered from insufficient experiments was deemed unreliable. Here, as noted by the Office, the Declarations provided by the Applicants are based upon ***repeat experiments*** of the prior art. As such, no "more extensive" experiments could be conducted. Applicants urge that a direct repeat of the experiments is dispositive. Indeed, *In re Michalek* does not indicate that a direct repeat of the experiments was performed, only that the

experiments were "within the disclosure." Moreover, the Declarations clearly state which experiments were repeated (see, e.g., September 30, 2002 Sæbo Declaration at paragraphs 3, 4, 5, 6) and provides the actual chromatography data from the experiments. Thus, there is no reason for the Office to "assume" anything about the data. The data and its import are clear on their face.

In particular, the Applicants followed the exact instructions of both Cook et al. and Cain et al. and analyzed the product. The Applicants did not fail to obtain CLA. Indeed, they obtained CLA with the isomers described by Cook et al. and Cain et al. However, the fact remains that the CLA also contained other isomers that are not mentioned by both Cook et al. and Cain et al. Cook et al.'s and Cain et al.'s silence concerning the presence of the isomers cannot be equated with the absence of the isomers. In particular, neither Cook et al. nor Cain et al. specifically define CLA to include non-active CLA isomers. As noted by Mr. Saebo:

> The results of the Cook repeat are generally in agreement with statements made by the inventors in Pariza, M.W., Y. Park, and M.E. Cook, "The biologically active isomers of conjugated linoleic acid," Progress in Lipids Research 40:283-298 (2001). At page 287, in section 2.2, they state: For example, CLA that we typically produce for experimental purposes consists of the *cis*-9,*trans*-11 (40.8-41.1%), *trans*-10,*cis*-12 (43.5-44.9%), and *trans*-9,-*trans*-11/*trans*-10-*trans*-12 (4.6-10%) isomers. This provides evidence that the CLA compositions of Cook and Pariza contained greater than 1% trans-trans isomers. Furthermore, the presence of the trans-trans isomers is indicative of greater than 1% of 11,13 and 8,10 isomers.

In response to the presentation of actual experimental evidence, the Office alleged, "[t]he Office is not convinced by the assumption that Cain et al. cannot detect the trans trans isomers, or simply ignore the presence of the isomers. The evidence provided with the declaration has been fully evaluated against the cited reference. Appellant provided his own results contrary to the references, making assumption without factual support. The evidences provided by the appellant fail to prove that, as a matter of fact, the data reported by Cain et al. or Cook et al. is

either incorrect or incomplete" (Answer to Appellant's Appeal Brief, page 7). These allegations are conclusory and do not provide any indication that the Office has actually evaluated or considered the evidence. Thus, the Applicant's results are not contrary to those reported in Cain et al. and Cook et al. The Applicant's results simply supplement Cain et al. and Cook et al. This is why Applicants' repeat experiments were necessary.

Regarding the inability of Cook et al. to detect levels of trans,trans 8,10 and 11,13 octadecadienoic acid isomers, the Office asserts, "GC method is a well-developed analytical method even in 1996. Method for CLA analysis may be further optimized later. However, there is no evidence showing GC method in 1996 were crude enough to miss those well-recognized CLA isomers" (Answer to Appellant's Appeal Brief, page 8). The Office is missing the point. Regardless of the state of technology, the fact remains that Cook et al. could not produce compositions comprising *less than* 2% trans,trans 8,10 and 11,13 octadecadienoic acid isomers.

Regarding the inability of Cain et al. to detect levels of trans,trans 8,10 and 11,13 octadecadienoic acid isomers, the Office asserts, "Cain et al. have recognized the existence of the trans, trans, 8,10- and 11,13- octadecadienoic isomers. (page 1, lines 15-25). Cain is not silent about the isomers identified herein, and therefore is not likely to simply ignore the present of those isomers" (Answer to Appellant's Appeal Brief, page 8). The Declarations are not stating that Cain et al. does not recognize the *existence* of trans, trans, 8,10- and 11,13- octadecadienoic isomers. Rather, the Declarations establish that Cain et al. *could not produce* compositions comprising less than 2% trans,trans 8,10 and 11,13 octadecadienoic acid isomers.

B. The Office Has Committed Reversible Error

The Office has dismissed the Applicant's arguments and the Sæbo Declarations by citing *In re Michalek* and then providing absolutely no other explanation as to why the data contained in the Declarations fail to rebut the Office's *prima facie* case of obviousness. This is reversible error under *In re Alton*, 76 F.3d 1168, 37 U.S.P.Q.2d 1578 (Fed. Cir. 1996).

In *In re Alton*, the applicants submitted a declaration in order to rebut a *prima facie* case of inadequate written description by the Board of Appeals in an earlier appeal. *Id.* at 1173. Instead of addressing the arguments presented in the declaration, the Office dismissed the declaration as opinion evidence that was entitled to little weight. *Id.* at 1173-745. The Federal Circuit remanded the case to the Board, holding that the Board committed error in both viewing the declaration as opinion evidence and dismissing the declaration "without an adequate explanation of why the declaration failed to rebut the Board's *prima facie* case" of unpatentability. Id. at 1174. These bases for reversal were independent. With respect failure to provide an adequate explanation of why the declaration failed to rebut the *prima facie* case, the Federal Circuit found that:

> In sum, the Office dismissed the Wall declaration and provided only conclusory statements as to why the declaration did not show that a person skilled in the art would realize that Alton had possession of the claimed subject matter in 1983.

Id. at 1176. In particular, the Federal Circuit held that the Office failed to address specific points made in the declaration concerning modifications of the amino acids sequence of protein. *Id.*

In re Alton is directly applicable to the present facts. Instead of addressing the factual evidence and arguments presented in the Saebo Declarations and Office Action Responses, the Office has dismissed the Declarations with the conclusory reasoning that it is entitled to "little weight" because it is a repeat of the prior art by an inventor. Thus, under *In re Alton*, the Office

has failed to meet the standard for replying to evidence presented in the Sæbo Declarations. In the Answer to Appellant's Appeal Brief, the Office does not address *In re Alton* and its holding of reversible error when a Declaration is not properly considered and evaluated. Accordingly, Applicants respectfully request that the Office's rejection be reversed.

C. The Sugano et al. Reference is Consistent with Applicant's Declaration

Additionally, other evidence not generated by the Applicants is consistent with the Applicants data. No chemical isomerization method has been described in the literature to date which does not produce a variety of isomers. Indeed, it is impossible to isomerize linoleic acid by the methods described in Cook et al. or Cain et al. without producing other isomers due to the process known as thermal sigmatropic rearrangement. This process is described in Chapter 5 of the book *Advances in Conjugated Linoleic Acid Research, Volume 2*, J. Sebedio, W.W. Christie, and R. Adolf, Eds., AOCS Press, Champaign, IL, 2002 (Attached hereto as Appendix C) and the Declarations attached at Appendix B. Mr. Saebo wrote this chapter. Briefly, the research described in this chapter establishes that the formation of the 8,10 and 11,13 isomers is a necessary consequence of heating compositions containing the t10,c12 and c9,t11 isomers. Thus, whenever compositions containing t10,c12 and c9,t11 CLA are heated at temperatures such as those used by Cook et al. (i.e., 180°C for 2.5 hours) and Cain et al. (i.e., 180°C for about 2 - 2.5 hours), 8,10 and 11,13 isomers are necessarily produced. Accordingly, it appears that both Cook et al. and Cain et al. simply failed to conduct an analysis for the other isomers present in the isomerized product since those isomers were necessarily present.

This conclusion is also supported by reference to Sugano et al., Lipids 33(5):521-527 (1998)(reference 47 in Form 1449 filed April 10, 2000, copy attached hereto at Appendix D for

the Office's convenience). Sugano et al. isomerized linoleic acid conditions similar to those described by Cain et al. The conditions utilized in the three references are compared in following Table.

Cain et al.	**Sugano et al.**
50 g linoleic acid, 95% pure	50 g linoleic acid, 99% pure
Solvent: 290 grams ethylene glycol	Solvent: 290 grams ethylene glycol
Catalyst: 15 g NaOH	Catalyst: 15 g NaOH
Reaction time: 2 hours	Reaction time: 2 hours
Reaction temperature: 180° C	Reaction temperature: 180° C
Reaction atmosphere: Inert	Reaction atmosphere: Nitrogen

As can be seen, the reaction conditions were almost identical. However, the results are not. As noted on page 522 of Sugano, the resulting CLA preparation contained the following CLA isomers: 29.8% c9,t11/t9,c11; 29.6% t10,c12; 1.3% c9,c11; 1.4% c10,c12; 18.6% t9,t11/t10,t12; 5.6% linoleic acid; and 13.7% other isomers. In contrast to the simplified analysis presented in both Cook et al. and Cain et al., isomerization of CLA results in the production of many different isomers, not just the desired c9,t11 and t10,c12 isomers.

The Office refutes the Sugano et al. results, and alleges, "it is noted that appellant thas recognized that the procedure in Sugano et al. is merely similar to, but not the same as, that disclosed in Cain et al. Many of the conditions have not been fully described, such as the way for heating or cooling, acid used to acidify the reaction mixture, the way to extract the product from the reaction mixture, the impurity in the employed materials, etc. all those conditions might

have well affected the final product. Having seen the results in Cain et al. and Sugano et al., one of ordinary skill in the art would not come to the conclusion that one of the results must be incorrect or incomplete, and/or, make a judgment on which data is correct. Further, it is noted that example 6 in Cain et al. employs sunflower oil as the source of linoleic acid, which was not used by Sugano et al." (Answer to Appellant's Appeal Brief, page 9).

The Office is missing the point being made by the Sugano et al. and Cain et al. comparison. In particular, the Declarations do not state or imply that Sugano et al. and Cain et al. involved identical experiments. Rather, the Declarations state that Sugano et al. and Cain et al. involved *very similar* reactions. The Office does not address the fact that Sugano et al. indicates that isomerization of CLA results in the production of many different isomers, not just the desired c9,t11 and t10,c12 isomers.

Indeed, the nearly identical reaction conditions between Cain et al. and Sugano et al. but extremely different results negates any argument that no other references beyond Cain et al. show that the isomers identified herein would be produced in significant amounts under Cain's conditions. The Office's statement that one of skill in the art would not need to come to a conclusion as to which of the data in the Cain et al. or Sugano et al. is correct is simply incredulous.

As further support for this fact, the Board's attention is respectfully directed to example 1 of the instant application. This example compares the effect of varying temperature and reaction duration on CLA yield and composition. **The important fact to note is that each reaction, even at low temperatures (Example 1, Table 1), produced a distribution of the expected isomers, not just the c9,t11 and t10,c12 isomers.** Thus, ample evidence supports the conclusion that the analysis of Cook et al. and Cain et al. is either incorrect or incomplete. Both

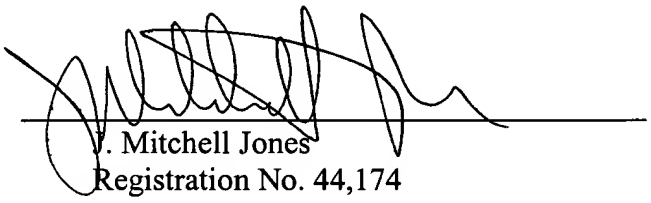
Cook et al. and Cain et al. appear to have either not analyzed for the isomers or chosen not to present data pertaining to the other isomers in their analysis. This is understandable because at the time, the other isomers were not expected to have a biological effect. However, just because data on these isomers was not presented does not mean that they are not present. Indeed, the evidence establishes that they were necessarily present as a result of the reaction conditions used by Cook et al. and Cain et al. **Thus, the compositions of Cook et al. and Cain et al. necessarily contained levels 8,10; 11,13; and trans,trans isomers that do not meet the claimed levels.** As such, the present invention is not obvious in light of Cook et al. and Cain et al.

Accordingly, Applicants respectfully request that this ground of rejection be removed and the claims passed to allowance.

B. Conclusion

For the foregoing reasons, it is submitted that the Office's rejection of Claims 1-6 and 8 was erroneous, and reversal of the rejection is respectfully requested. Appellant requests either that the Board render a decision as to the allowability of the claims, or alternatively, that the application be remanded for reconsideration by the Office.

Dated: December 6, 2004



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APPENDIX A

CLEAN VERSION OF THE ENTIRE SET OF PENDING CLAIMS

1. (previously presented) A food product comprising conjugated linoleic acid alkyl esters in a biologically active concentration, said alkyl esters comprising less than about two percent trans,trans; 8,10 and 11,13 octadecadienoic acid isomers.

2. (previously presented) The food product of claim 1 wherein the concentration of conjugated linoleic acid alkyl esters in said food product is about 0.05 to 3.5 percent by weight.

3. (previously presented) The food product of claim 1 wherein said conjugated linoleic acid alkyl ester is comprised of at least 50 percent up to about 99 percent by weight of octadecadienoic acid alkyl ester isomers selected from the group consisting of c9,t11-octadecadienoic acid alkyl ester and t10,c12-octadecadienoic acid alkyl ester.

4. (previously presented) A conjugated linoleic acid alkyl ester composition for safe use as a feed, food ingredient, or food supplement obtained by direct isomerization of an unrefined linoleic acid comprising

a composition of isomers in one part comprising at least 50 percent by weight of ester isomers selected from the group consisting of c9,t11- octadecadienoic acid alkyl ester and t10,c12-octadecadienoic acid alkyl ester, and combinations thereof, and

in a second part comprising less than two percent by aggregate weight of ester isomers selected from the group consisting of 8,10-octadecadienoic acid alkyl esters, 11,13-octadecadienoic acid alkyl esters, and trans,trans-octadecadienoic acid alkyl esters, and

in a third part comprising in the range of 0.1 to 0.5 percent phosphatidyl residue remaining after isomerization of said unrefined linoleic acid.

5. (previously presented) The ester composition of claim 4 wherein said c9,t11-octadecadienoic acid alkyl ester contained in said first composition part constitutes greater than 60 percent of the total isomers of octadecadienoic acid alkyl esters.

6. (previously presented) The ester composition of claim 4 wherein said t10,c12-octadecadienoic acid alkyl ester contained in said first composition part constitutes greater than 60 percent of the total isomers of octadecadienoic acid alkyl esters.

7. (Canceled).

8. (previously presented) The food product of claim 1 wherein said conjugated linoleic acid alkyl esters have an alkyl radical selected from the group consisting of methyl-, ethyl-, propyl-, isopropyl-, butyl-, and isobutyl-.

APPENDIX B

Declarations of Asgeir Saebo with attachments

PATENT
Attorney Docket No. **CONLINCO-03681**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Asgeir Sæbo *et al.*		
Serial No.:	09/271,024	Group No.:	1617
Filed:	03/17/99	Examiner:	Wang
Entitled:	**CONJUGATED LINOLEIC ACID COMPOSITIONS**		

Declaration of Asgeir Sæbo

Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated: 12/13/2002 By: Susan M. McClintock
Susan M. McClintock

I, Asgeir Sæbo, state as follows:

1. My present position is Director of Research, Natural AS.

2. I have reviewed the above captioned patent application, of which I am an inventor, the Office Action mailed August 13, 2002, and the Cook, Nilsen and Pariza references cited as prior art.

3. I have conducted repeats of the conjugation methods described in WO97/18320 to Cain.

4. In the repeat of Cain, the conjugation conditions were the same as those described in Example 6 of WO97/18320. The results of the conjugation reactions were analyzed by GC-MS. The results are attached at Tab 1. As can be seen, this conjugation method resulted in a conjugated linoleic acid composition comprising approximately 3.49% c11,t13 CLA and 2.24% t9,t11and t10,t12 CLA. The t8,c10 isomer co-elutes with the c9,t11 isomers, but almost always occurs in a one to one proportion to the c11,t13 isomer. I note that this method is very similar to the method utilized in the Sugano reference, which was discussed in my previous Declaration.

My work confirms that these methods produce CLA with relatively high levels of undesirable isomers.

5. The Examiner states at page 3 of the Office Action that Cain teaches CLA compositions that are composed of 48.9% c9,t11 and 51.1% t10,c12 CLA, and that the analysis was carried out with gas chromatography and no other isomer of conjugated linoleic acid is detected. However, this does not mean that the other isomers were not present, as was found in my repeat of Cain. This discrepancy is explainable by the facts that 1) methods for the analysis of CLA compositions in 1996 were rather crude and 2) Cain may have simply chosen not to include non-active isomers when reporting their results. Improved methods for detecting the various isomers of CLA were not developed until well after the 1995 priority date of Cain. This fact is substantiated by Yurawecz *et al.* (attached at Tab 2), who state "the CLA products analyzed in this study were found to contain up to 12 geometric and positional CLA isomers. These findings are based on appropriate and improved analytical methodologies [including gas chromatography techniques] that have only recently been developed." (Yurawecz, *p.* 281). Thus, Cain et al. may not have conducted an analysis which could detect the isomers in questions. Consideration of Example 18 of Cain et al. supports this analysis. The inventors state that their compositions, produced by the method of Example 6, contained 63.8% CLA, of which 48.9% was the cis 9, trans 10 isomer and 51.1% was the trans 10, cis 12 isomer. This means that the inventors provide no analysis of the remaining 36.2% of their composition. The 8,10; 11,13; and trans-trans isomers that are discriminated against in the present invention and detected in my repeat of Cain could well have been present in this fraction.

6. With respect to the Nilsen reference, I note that it does not provide any method of producing conjugated linoleic acid having less than 1% 8,10; 11,13; and trans-trans isomers.

7. With respect to the Pariza application, I note that the passages cited by the Examiner (column 4, line 50, bridging column 8, line 68) do not teach preparation of CLA in amounts suitable for incorporation into acylglycerides. Instead, the HPLC purified isomers are produced only for use as chromatography standards. Furthermore, Pariza does not disclose using the purified isomers for any other use but as standards. In other words, Pariza does not disclose using the purified isomers to prepare acylglycerides or food products, or using a combination of (i.e., c9,t11 and t10,c12) purified isomers in any product.

8. I further understand that the Examiner has requested evidence of the criticality, or

unexpected benefit of CLA compositions containing less than 1% of 8,10; 11,13, and trans-trans octadecadienoic acid isomers. I refer the Examiner to the publication attached at Tab 2, Yurawecz et al., Variation in isomer distribution in commercially available conjugated linoleic acid, Fett/Lipid 101:277-282 (1999). This study, by researchers at the U.S. Food and Drug Administration (U.S.F.D.A.), was "undertaken to determine the content and distribution of CLA isomers in commercially available CLA capsules and liquid products with labels stating to contain CLA." In brief, the authors of the Yurawecz *et al.* publication note that:

> While it has not been established, which isomer(s) is (are) responsible for the reported beneficial properties of CLA, it is generally thought that anticarcinogenicty is due to rumenic acid [c9,t11 octadecadienoic acid]. The nutritional and physiological effects, if any, of other CLA isomer(s) in commercially available CLA preparations are not known.

(Yurawecz, *p.* 280). In an additional reference cited within Yurawecz *et al.*, (published by members of the Yurawecz group) it was found that the 11 *cis*, 13 *trans*-18:2 isomer was found to was found to accumulate preferentially in heart phospholipids and specifically in heart and liver diphosphatidylglycerol (DPG) of pigs feed commercial CLA mixtures. Yurawecz *et al.* note that in response to their "findings that 11 *cis*, 13 *trans*-18:2 was selectively incorporated into DPG . . . , a major supplier of commercial CLA preparations recently modified [their production] process to eliminate the 11 *cis*, 13 *trans*-18:2 isomer." (Yurawecz, *p.* 281). Thus, it is desirable to control the amounts of CLA isomers of unknown function in CLA compositions.

9. This conclusion is also supported by Adlof et al., Changes in Conjugated Linoleic Acid Composition Within Samples Obtained from a Single Source, Lipids 36(3):315-17 (2001), attached hereto at Tab 3. At page 315, the authors state:

> If indeed certain daily levels of CLA intake are required to produce suggested health benefits in humans, changes in concentrations of specific CLA isomers could significantly impact these effects. Care must be taken to analyze the CLA used in human and animal studies.

10. I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

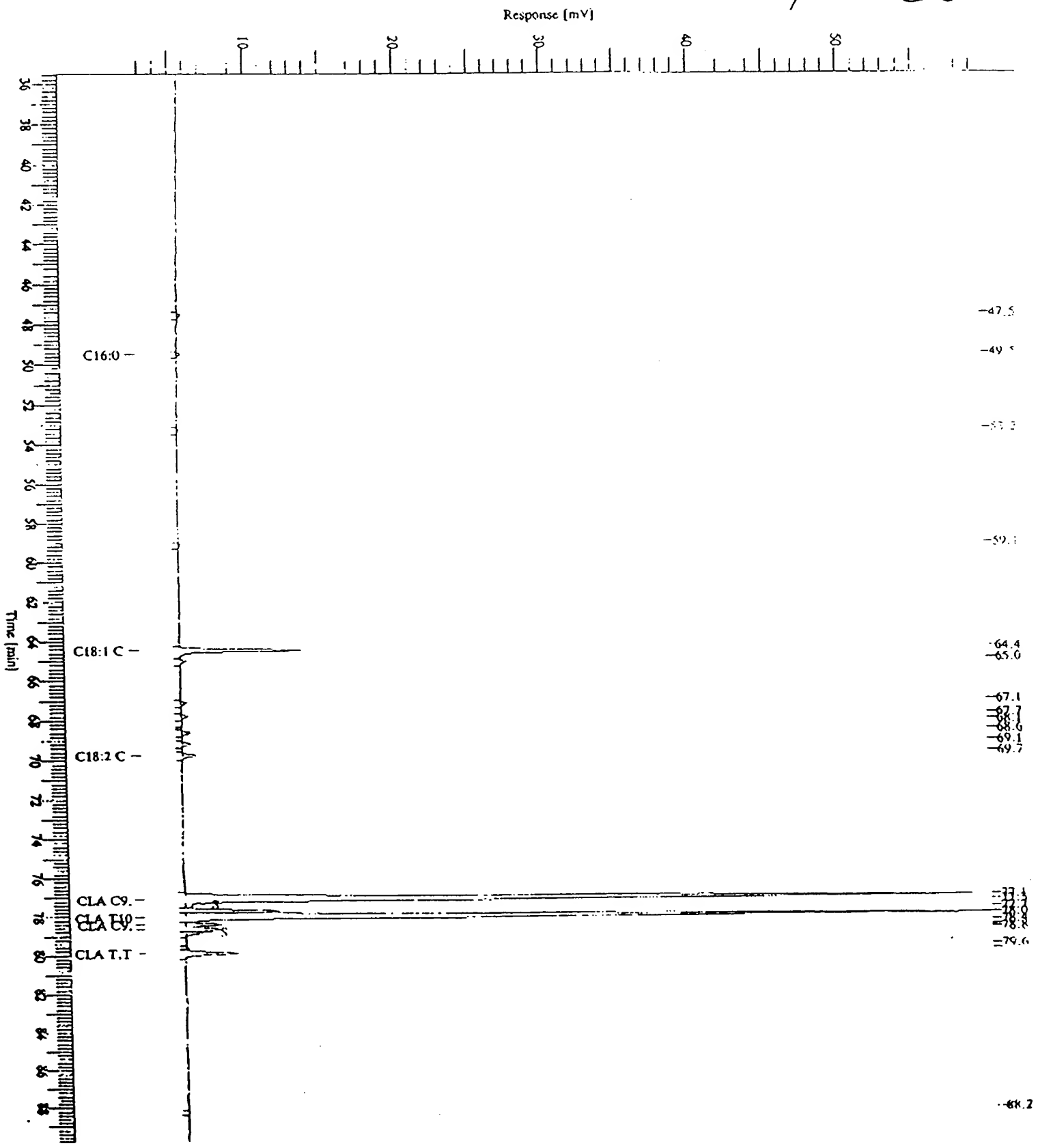
PATENT
Attorney Docket No. **CONLINCO-03681**

Asgeir Sæbo

Date: Dec. 10. 2002

Sample Name : 6659: A01348, 024/96-1, CLA FPA Sample #: 001 Page 1 of 1
FileName : D:\TCWS Data\data\Data 100E 1000-15_\100e1001.raw
Date : 19.11.01 11:48:38
Method : Time of Injection: 19.11.01 09:08:32
Start Time : 35.53 min End Time : 89.83 min Low Point : 2.75 mV High Point : 59.51 mV
Plot Offset: 2.75 mV Plot Scale: 56.8 mV

WO97/18320



Software Version : 6.1.2.0.1:D19
Sample Name : 6659: A01348, 024/96-1, CLA FFA
Instrument Name : GC
Rack/Vial : 0/1
Sample Amount : 1.000000
Cycle : 1
Date : 19.11.01 11:48:37
Data Acquisition Time : 19.11.01 09:08:32
Channel : B
Operator : Operatør
Dilution Factor : 1.000000

Result File : D:\TCWS Data\data\Data 100E 1000-1999\100e1001.rst
Sequence File : D:\TCWS Data\sekvenser\100E.20.10.00..seq

FATTY ACID PROFILE REPORT

PERKIN ELMER AUTOSYSTEM XL GC

Column: WCOT FUSED SILICA 100 m x 0.25 mm COATING CP-SIL 88 DF= 0.2 Chrompack cat.no: 7489
Carrier Gas: He, 30.0 PSI
Method: 100E.mth
Temp: 80 C (2 min)->45 C/ min->130 C (0 min)->1 C/ min-> 220 C (10 min)
Injection: Splitless, 240 C
Detector: FID, 280 C

Peak #	Time [min]	Component Name	Area [%]	Area [µV·s]	Height [µV]
1	47.557		0.14	2040.57	221.66
2	49.507	C16:0	0.12	1770.08	234.26
3	53.277		0.07	1043.10	118.41
4	59.139		0.07	1079.52	131.55
5	64.461	C18:1 c9	4.84	72109.91	8053.81
6	65.035		0.23	3435.33	396.61
7	67.125		0.25	3718.15	401.86
8	67.795		0.28	4195.57	459.60
10	68.621		0.31	4688.64	520.82
11	69.176		0.33	4880.16	532.98
12	69.744	C18:2 c9,c12	0.53	7977.36	868.60
13	77.128	CLA c9,t11+t8,c10	42.84	638739.60	52812.75
14	77.371		0.28	4120.52	216.07
15	77.752	CLA c11,t13	3.49	51987.22	6233.41
16	78.067	CLA t10,c12	40.35	601682.23	54289.00
17	78.437	CLA c9,c11	1.36	20327.77	2373.19
18	78.664	CLA c10,c12	1.61	24007.50	2280.68
19	78.808		0.58	8661.37	1107.38
20	79.693		0.08	1265.48	173.63
21	79.909	CLA t,t 9,11+10,12	2.24	33420.59	3512.11
			100.00	1491150.67	134938.38

Missing Component Report

Component	Expected Retention (Calibration File)
C18:0	0.001

19.11.01 11:48:37 Result: D:\T\ /S Data\data\Data 100E 1000-1999\100e1001.rst

Analyzed by: Natural ASA, Hovdebygda

Approved by: _____

ISSN 0931-5985
Volume 101 (8) 271–312

Fett
Zeitschrift für Wissenschaft und Technologie der Fette, Öle und Wachse

Lipid
Journal for Science and Technology of Fats, Oils and Waxes

Inhalt

Contents

PATENT
Attorney Docket No. **CONLINCO-04286**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Asgeir Sæbo *et al.*		
Serial No.:	09/132,593	Group No.:	1615
Filed:	08/11/98	Examiner:	Wang
Entitled:	**CONJUGATED LINOLEIC ACID ALKYLESTERS IN FEED STUFFS AND FOOD**		

Declaration of Asgeir Sæbo

Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated: By: Mary Ellen Warke

I, Asgeir Sæbo, state as follows:

1. My present position is Director of Research, Natural AS.

2. I have reviewed the above captioned patent application, of which I am an inventor, the Office Action mailed April 10, 2002, and the Cook, Baltes, Cain and Chin references cited as prior art.

3. I have conducted repeats of the conjugation methods described in U.S. Pat. No. 5,554,646 to Cook and WO97/18320 to Cain.

4. In the repeat of Cook, the conjugation conditions were the same as those described in Column 2 of U.S. Pat. No. 5,554,646. The results of the conjugation reactions were analyzed by GC-MS. The results are attached at Tab 1. As can be seen, this conjugation method resulted in in a conjugated linoleic acid composition comprising approximately 1.58% c11,t13 CLA and 2.34% t9,t11and t10,t12 CLA. The t8,c10 isomer co-elutes with the c9,t11 isomers, but almost always occurs in a one to one proportion to the c11,t13 isomer.

5. The results of the Cook repeat are generally in agreement with statements made by the inventors in Pariza, M.W., Y. Park, and M. E. Cook, "The biologically active isomers of conjugated linoleic acid," Progress in Lipids Research 40:283-298 (2001). At page 287, in section 2.2, they state:

> For example, CLA that we typically produce for experimental purposes consists of the *cis*-9,*trans*-11 (40.8-41.1%), *trans*-10,*cis*-12 (43.5-44.9%), and *trans*-9,-*trans*-11/*trans*-10-*trans*-12 (4.6-10%) isomers.

This provides evidence that the CLA compositions of Cook and Pariza contained greater than 1% trans-trans isomers. Furthermore, the presence of the trans-trans isomers is indicative of greater than 1% of 11,13 and 8,10 isomers.

6. In the repeat of Cain, the conjugation conditions were the same as those described in Example 6 of WO97/18320. The results of the conjugation reactions were analyzed by GC-MS. The results are attached at Tab 2. As can be seen, this conjugation method resulted in a conjugated linoleic acid composition comprising approximately 3.49% c11,t13 CLA and 2.24% t9,t11and t10,t12 CLA. The t8,c10 isomer co-elutes with the c9,t11 isomers, but almost always occurs in a one to one proportion to the c11,t13 isomer. I note that this method is very similar to the method utilized in the Sugano reference, which was discussed in my previous Declaration. My work confirms that these methods produce CLA with relatively highj levels of undesirable isomers.

7. The Examiner states at page 7 and 8 of the Office Action that Cain teaches CLA compositions that are composed of 48.9% c9,t11 and 51.1% t10,c12 CLA, and that the analysis was carried out with gas chromatography and no other isomer of conjugated linoleic acid is detected. However, this does not mean that the other isomers were not present, as was found in my repeat of Cain. This discrepancy is explainable by the facts that 1) methods for the analysis of CLA compositions in 1996 were rather crude and 2) Cain may have simply chosen not to include non-active isomers when reporting their results. Improved methods for detecting the various isomers of CLA were not developed until well after the 1995 priority date of Cain. This fact is substantiated by Yurawecz *et al.* (attached at Tab 3), who state "the CLA products analyzed in this study were found to contain up to 12 geometric and positional CLA isomers. These findings are based on appropriate and improved analytical methodologies [including gas chromatography techniques] that have only recently been developed." (Yurawecz, *p.* 281). Thus, Cain et al. may not have conducted an anlysis which could detect the isomers in questions. Consideration of Example 18 of Cain et al. supports this analysis. The inventors state that their

compositions, produced by the method of Example 6, contained 63.8% CLA, of which 48.9% was the cis 9, trans 10 isomer and 51.1% was the trans 10, cis 12 isomer. This means that the inventors provide no analysis of the remaining 36.2% of their composition. The 8,10; 11,13; and trans-trans isomers that are discriminated against in the present invention and detected in my repeat of Cain could well have been present in this fraction.

8. I further understand that the Examiner has requested evidence of the criticality, or unexpected benefit of CLA compositions containing less than 2% of 8,10; 11,13, and trans-trans octadecadienoic acid isomers. I refer the Examiner to the publication attached at Tab 3, Yurawecz et al., Variation in isomer distribution in commercially available conjugated linoleic acid, Fett/Lipid 101:277-282 (1999). This study, by researchers at the U.S. Food and Drug Administration (U.S.F.D.A.), was "undertaken to determine the content and distribution of CLA isomers in commercially available CLA capsules and liquid products with labels stating to contain CLA." In brief, the authors of the Yurawecz *et al.* publication note that:

> While it has not been established, which isomer(s) is (are) responsible for the reported beneficial properties of CLA, it is generally thought that anticarcinogenicty is due to rumenic acid [c9,t11 octadecadienoic acid]. The nutritional and physiological effects, if any, of other CLA isomer(s) in commercially available CLA preparations are not known.

(Yurawecz, *p.* 280). In an additional reference cited within Yurawecz *et al.*, (published by members of the Yurawecz group) it was found that the 11 *cis*, 13 *trans*-18:2 isomer was found to was found to accumulate preferentially in heart phospholipids and specifically in heart and liver diphosphatidylglycerol (DPG) of pigs feed commercial CLA mixtures. Yurawecz *et al.* note that in response to their "findings that 11 *cis*, 13 *trans*-18:2 was selectively incorporated into DPG . . . , a major supplier of commercial CLA preparations recently modified [their production] process to eliminate the 11 *cis*, 13 *trans*-18:2 isomer." (Yurawecz, *p.* 281). Thus, it is desirable to control the amounts of CLA isomers of unknown function in CLA compositions.

9. This conclusion is also supported by Adlof et al., Changes in Conjugated Linoleic Acid Composition Within Samples Obtained from a Single Source, Lipids 36(3):315-17 (2001), attached hereto at Tab 4. At page 315, the authors state:

> If indeed certain daily levels of CLA intake are required to produce suggested health benefits in humans, changes in concentrations of specific CLA isomers could significantly impact these effects. Care must be taken to analyze the CLA used in human and animal studies.

10. I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Asgeir Sæbo

Date: Sept. 30th. 2002

Sample Name : 6645:A01346, CLA FPA, B 025/34 Sample #: 001 Page 1 of 1
FileName : D:\TCWS Data\data\Data 100E\100e990...w
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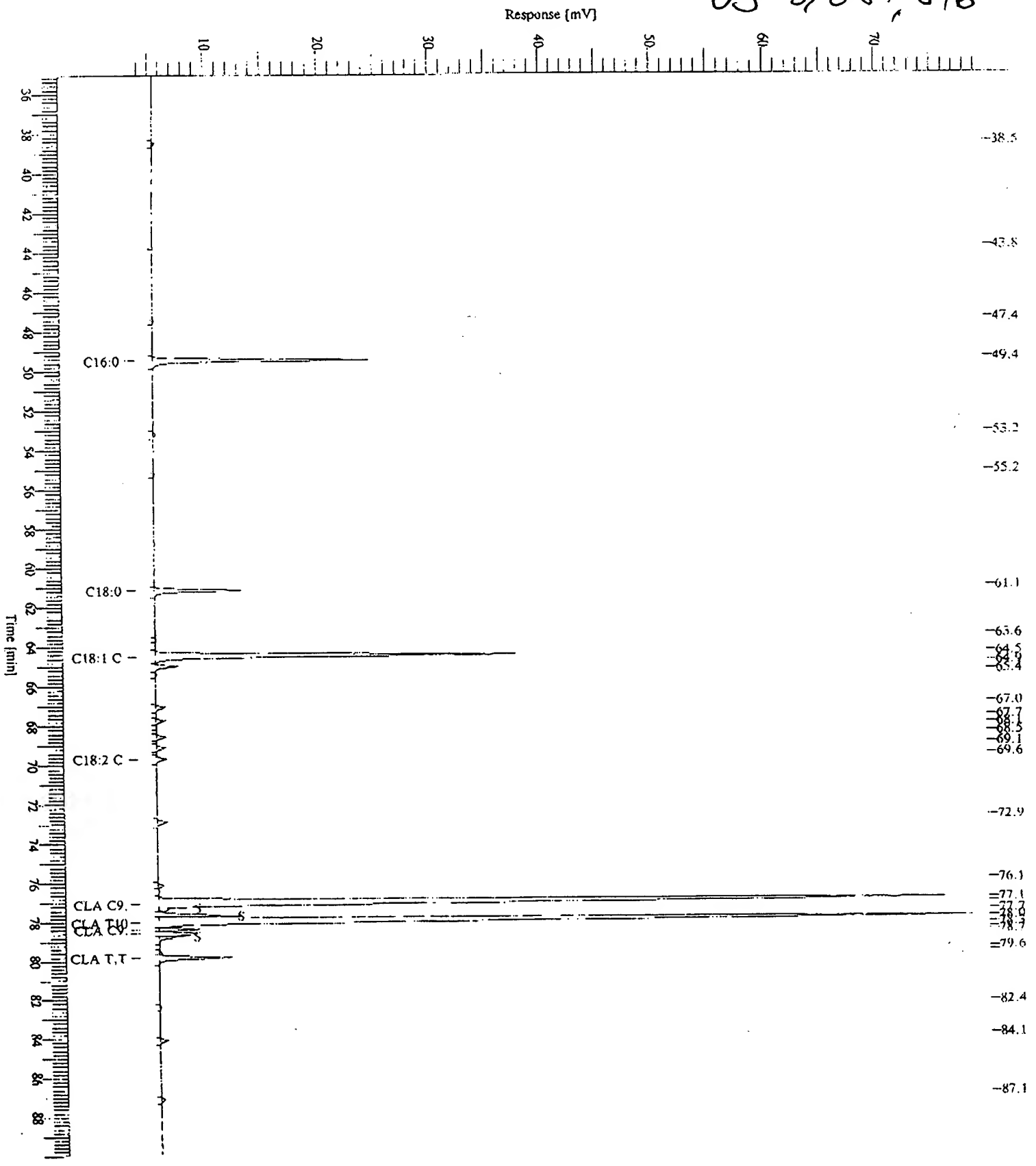
Method :
Time of Injection: 15.11.01 15:54:20
Start Time : 35.11 min End Time : 90.03 min Low Point : 3.70 mV High Point : 79.77 mV
Plot Offset: 3.70 mV Plot Scale: 76.1 mV

US 5,554,646



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Cycle : 1
Date : 16.11.01 10:29:05
Data Acquisition Time : 15.11.01 15:54:20
Channel : B
Operator : Operatør
Dilution Factor : 1.000000

Result File : D:\TCWS Data\data\Data 100E\100e990.rst
Sequence File : D:\TCWS Data\sekvenser\100E.20.10.00..seq

FATTY ACID PROFILE REPORT

PERKIN ELMER AUTOSYSTEM XL GC

Column: WCOT FUSED SILICA 100 m x 0.25 mm COATING CP-SIL 88 DF= 0.2 Chrompack cat.no: 7489
Carrier Gas: He, 30.0 PSI
Method: 100E.mth
Temp: 80 C (2 min)->45 C/ min->130 C (0 min)->1 C/ min-> 220 C (10 min)
Injection: Splitless, 240 C
Detector: FID, 280 C

Peak #	Time [min]	Component Name	Area [%]	Area [μV·s]	Height [μV]
1	38.515		0.09	2240.70	289.83
4	49.483	C16:0	6.41	165319.20	19173.70
5	53.229		0.08	2103.22	225.47
7	61.163	C18:0	2.66	68542.43	7691.48
9	64.509	C18:1 c9	12.74	328361.33	32287.86
10	64.984		0.67	17379.46	2043.14
11	65.416		0.04	1072.35	124.54
12	67.069		0.32	8202.39	896.28
13	67.747		0.30	7679.15	865.45
15	68.560		0.30	7795.39	852.42
16	69.120		0.29	7526.09	835.25
17	69.680	C18:2 c9,c12	0.34	8668.83	903.73
18	72.917		0.36	9200.84	981.82
19	76.131		0.17	4277.85	514.27
20	77.099	CLA c9,t11	34.16	880864.80	70319.32
21	77.288		0.24	6213.92	234.34
22	77.704	CLA c11,t13	1.58	40803.24	5407.69
23	78.045	CLA t10,c12	33.54	864675.26	72871.17
24	78.384	CLA c9,c11	1.15	29552.24	3582.74
25	78.605	CLA c10,c12	1.36	35038.09	3344.41
26	78.757		0.41	10511.86	1445.22
27	79.627		0.06	1626.11	240.27
28	79.851	CLA t,t 9,11+10,12	2.34	60437.73	6462.11
29	82.445		0.04	1128.27	115.02
30	84.131		0.23	6040.92	748.16
31	87.141		0.12	3122.78	358.28
			100.00	2578384.48	232813.97

16.11.01 10:29:05 Result: D:\T[illegible]S Data\data\Data 100E\100e990.rst

Missing Component Report

Component	Expected Retention (Calibration File)

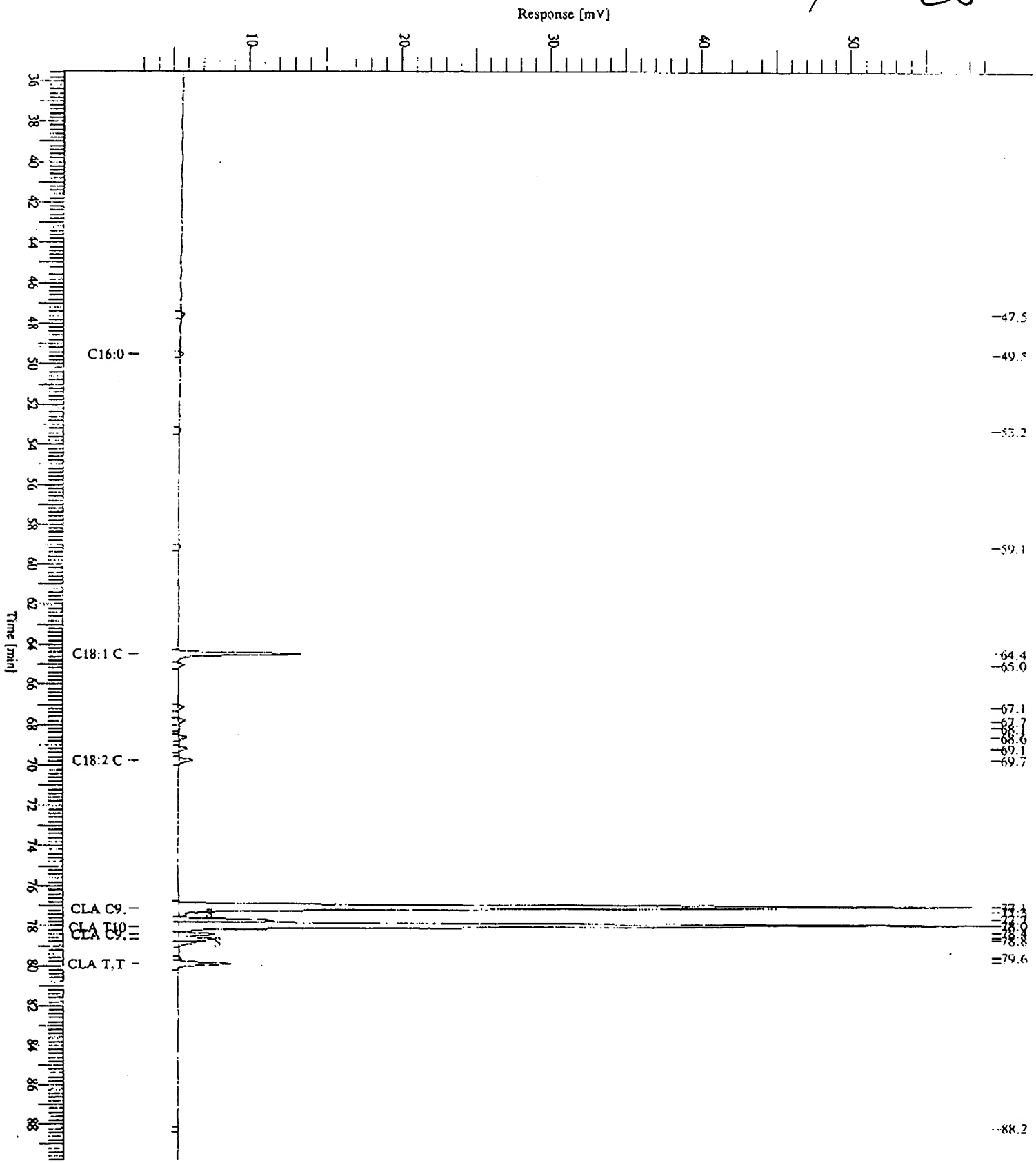
All components were found

Analyzed by: Natural ASA, Hovdebygda

Approved by: _____

Sample Name : 6659: A01348, 024/96-1, CLA FFA Sample #: 001 Page 1 of 1
FileName : D:\TCWS Data\data\Data 100E 1000-19..\100e1001.raw
Date : 19.11.01 11:48:38
Method : Time of Injection: 19.11.01 09:08:32
Start Time : 35.53 min End Time : 89.83 min Low Point : 2.75 mV High Point : 59.51 mV
Plot Offset: 2.75 mV Plot Scale: 56.8 mV

WO 97/18320



Software Version : 6.1.2.0.1:D19
Sample Name : 6659: A01348, 024/96-1, CLA FFA
Instrument Name : GC
Rack/Vial : 0/1
Sample Amount : 1.000000
Cycle : 1
Date : 19.11.01 11:48:37
Data Acquisition Time : 19.11.01 09:08:32
Channel : B
Operator : Operatør
Dilution Factor : 1.000000

Result File : D:\TCWS Data\data\Data 100E 1000-1999\100e1001.rst
Sequence File : D:\TCWS Data\sekvenser\100E.20.10.00..seq

FATTY ACID PROFILE REPORT

PERKIN ELMER AUTOSYSTEM XL GC

Column: WCOT FUSED SILICA 100 m x 0.25 mm COATING CP-SIL 88 DF= 0.2 Chrompack cat.no: 7489
Carrier Gas: He, 30.0 PSI
Method: 100E.mth
Temp: 80 C (2 min)->45 C/ min->130 C (0 min)->1 C/ min-> 220 C (10 min)
Injection: Splitless, 240 C
Detector: FID, 280 C

Peak #	Time [min]	Component Name	Area [%]	Area [µV·s]	Height [µV]
1	47.557		0.14	2040.57	221.66
2	49.507	C16:0	0.12	1770.08	234.26
3	53.277		0.07	1043.10	118.41
4	59.139		0.07	1079.52	131.55
5	64.461	C18:1 c9	4.84	72109.91	8053.81
6	65.035		0.23	3435.33	396.61
7	67.125		0.25	3718.15	401.86
8	67.795		0.28	4195.57	459.60
10	68.621		0.31	4688.64	520.82
11	69.176		0.33	4880.16	532.98
12	69.744	C18:2 c9,c12	0.53	7977.36	868.60
13	77.128	CLA c9,t11+t8,c10	42.84	638739.60	52812.75
14	77.371		0.28	4120.52	216.07
15	77.752	CLA c11,t13	3.49	51987.22	6233.41
16	78.067	CLA t10,c12	40.35	601682.23	54289.00
17	78.437	CLA c9,c11	1.36	20327.77	2373.19
18	78.664	CLA c10,c12	1.61	24007.50	2280.68
19	78.808		0.58	8661.37	1107.38
20	79.693		0.08	1265.48	173.63
21	79.909	CLA t,t 9,11+10,12	2.24	33420.59	3512.11
			100.00	1491150.67	134938.38

Missing Component Report

Component	Expected Retention (Calibration File)
C18:0	0.001

19.11.01 11:48:37 Result: D:\T /S Data\data\Data 100E 1000-1999\100e1001.rst

Analyzed by: Natural ASA, Hovdebygda

Approved by: _____

APPENDIX C

Chapter 5 of the book Advances in *Conjugated Linoleic Acid Research, Volume 2*, J. Sebedio, W.W. Christie, and R. Adolf, Eds., AOCS Press, Champaign, IL, 2002

Advances in Conjugated Linoleic Acid Research, Volume 2

Editors

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Chapter 5

Commercial Synthesis of Conjugated Linoleate

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Introduction

Conjugated linoleic acid (CLA) has been available as a health food supplement in soft gelatine capsules since 1995 in the United States, and more recently in several European countries and Japan. CLA products designed for food and animal feed additives are expected to appear on the market in the near future. CLA has been produced for decades for technical purposes and continues to be used as a substitute for Chinese tung oil in the paint and varnish industry due to its "drying" characteristics. The production methods developed for technical CLA products were rapidly modified and improved upon after the discovery of the biological activity of the substance. This chapter will focus on supplements in particular, including current production methods, stability, and breakdown products. Purified isomers are currently available only for research purposes, but a few references to methods available for purification will be given.

CLA for Technical Applications

Dehydration of Ricinoleic Acid

Several decades ago, only two natural oils (tung oil and oiticica) were known to contain conjugated double bonds. Oils that contain these bonds rapidly form a polymer film ("drying") if a thin layer is exposed to air; tung oil was widely used in the paint and varnish industry. An increasing demand for such oils and limited availability encouraged efforts to produce drying oils from nonconjugated oils.

The main constituent of castor bean oil is ricinoleic acid (12-hydroxy-9-octadecenoic acid). Around 1937, dehydrated castor oil appeared on the market in the United States as a substitute for tung oil. Ten years later the product was established as one of the most popular drying oils (1). It has been known since 1888 that castor oil could be dehydrated, and since 1914 it was known that the main isomers of linoleic acid formed had double bonds at positions 9,11 and 9,12, but the detailed composition of dehydrated ricinoleic acid was not investigated until recently. A German patent from 1930 (2) and a U.S. patent from 1934 (3) describe the preparation of dehydrated castor bean oils. A modified procedure was recently used to produce an 83% pure 9-*cis*,11-*trans* CLA concentrate from purified ricinoleic acid (4). Main impurities were the 9-*cis*,11-*cis* and 9-*cis*,12-*trans*-octadecadienoic acids. Conventional dehydration

using high temperatures will create other isomers, mainly 8-*trans*,10-*cis* and *trans*, *trans* isomers. CLA from dehydrated castor oil is not available on the market in supplement form. Apart from safety issues, the reason is the absence of 10-*trans*,12-*cis* CLA, the isomer shown to inhibit fat synthesis (5).

Alkali Isomerization of Linoleic Acid Oils

Attempts to produce drying oil from nonconjugated oils were successful in the late 1930s as well as for oils containing methylene-interrupted fatty acids. In 1941, a U.S. patent was issued that describes the use of monohydric and polyhydric alcohols as solvents and a variety of alkaline catalysts (6). A few years later, two patents were issued that described the use of water (7) and steam (8), respectively, as solvent in an autoclave to achieve the temperatures necessary to conjugate unsaturated acids. It is actually the soap that is conjugated. Upon addition of mineral acid, the conjugated free fatty acids are liberated. Currently, CLA is produced for technical purposes in high alkaline water at ~230°C. Feedstock is usually free fatty acids (after fat splitting to recover glycerol). The product is usually distilled to yield a virtually colorless oil.

Production of CLA for Animal and Human Consumption

Alkaline Water Isomerization

The first products to appear on the health food market contained ~65% CLA, and the profile of the CLA isomers was similar to technical-grade products. Christie *et al.* (9), showed that the main isomers of CLA in addition to 9-*cis*,11-*trans* and 10-*trans*,12-*cis* were an 8,10 and an 11,13 isomer *cis,trans* or *trans,cis*. These were later identified as 8-*trans*,10-*cis* and 11-*cis*,13-*trans* (10). Such products are still available as supplements, and most if not all are produced from linoleate-rich starting materials in high-alkaline water reactions at temperatures >230°C. We investigated reaction parameters in water alkaline (KOH or NaOH catalyst) reactions trying to avoid formation of 11-*cis*,13-*trans* and 8-*trans*,10-*cis*. It turned out not to be possible to achieve a nearly quantitative isomerization and at the same time avoid formation of the said isomers (data not published).

Isomerization in Propylene Glycol

Quantitative isomerization of oils containing polyunsaturated fatty acids in monohydric and polyhydric alcohols was described in 1941 (6). A detailed procedure using ethylene glycol is described in a patent from 1996 (11). Ethylene glycol has not been used commercially for production of CLA for consumer safety reasons. Propylene glycol has therefore been selected by several producers who independently developed proprietary procedures (12,13). KOH was selected as catalyst because of its high solubility compared with NaOH. Reaction temperatures are typically 130–180°C, and times of reaction are from 3 to >24 h. The quantity of KOH

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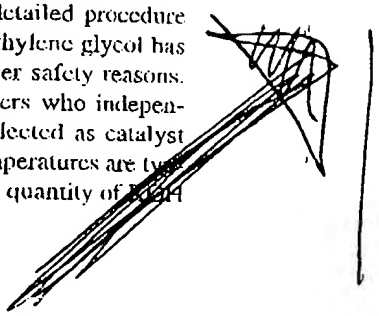


is substantial and in excess of that needed for quantitative saponification. After the reaction is complete, the mixture is cooled down and water and mineral acid (hydrochloric or sulfuric) are added. Free fatty acids of CLA are liberated as soon as the mixture becomes acidic. One patent describes the use of hexane at this point to extract CLA and facilitate separation from the bottom aqueous layer without emulsion problems. However, the operation is possible without the use of hexane. For the sake of recovery of propylene glycol, free fatty acids are preferred as feedstock oil. A triacylglycerol feedstock will create glycerol to contaminate the propylene glycol. After water and solvent (hexane if used) have been removed under vacuum, the CLA product is preferably purified by deodorization and distillation. Peroxides and volatiles are easily removed by deodorization. The peroxides are broken down to secondary volatile products that are removed in the process.

The purification process should also include a molecular distillation step to remove nonvolatile compounds such as polymers, sterols, and propylene glycol esters. Heavy metals could also arise from the isomerization process if mineral acids are used in stainless steel reactors (14). Their concentrations are reduced upon molecular distillation as well. A distilled product is almost colorless and has an acid value of ~200 (mg KOH/g). A nondistilled product might have an acid value of ~190, be yellow to slightly brown in color and have an opaque appearance. However, we have observed a slight decrease in acid value in capsules over time and also a darkening of the oil if the capsule material is colored. Due to the strong alkaline process, free fatty acids are the final product regardless of the form of feedstock (free fatty acid, a monoalkyl ester, or a triacylglycerol oil). Therefore, CLA in supplements are offered almost exclusively as free acids, in contrast to n-3 concentrates that are offered either as ethyl esters or reesterified triacylglycerols.

Isomerization of Mono-Alkyl Esters Using Alkali Metal Alcoholates

Recently, a proprietary method has been developed that quantitatively isomerizes methyl esters and ethyl esters of linoleic acid using very low quantities of catalysts and virtually no solvents (data not published). Because of the quantity of catalyst (~2%), only a small fraction of the ester is saponified and hence appears as free fatty acid after addition of a neutralizing agent. Most of the product (>92%) is still in the form of the methyl or ethyl ester after the isomerization process. The reaction proceeds at temperatures down to below 100°C, and the CLA product is characterized by very low levels of CLA isomers produced by thermal [1,5] sigmatropic rearrangements (see below).

Thermal [1,5] Sigmatropic Rearrangements of CLA Isomers

Production of CLA in propylene glycol or other alcohol under mild conditions gives rise to <0.5% each of the isomers 11-*cis*,13-*trans* and 8-*trans*,10-*cis*. After purification of single isomers, we showed that upon heating to 220°C in an inert atmosphere, 10-*trans*,12-*cis* gives rise to 11-*cis*,13-*trans* (Fig. 5.1). Upon heating an 11-*cis*,13-*trans* concentrate, 10-*trans*,12-*cis* was produced. Under optimal condi-

tions, an equilibrium is established between these isomers, and only minor quantities of *cis,cis* and *trans,trans* isomers are formed. The isomer shift is actually a thermal [1,5] sigmatropic rearrangement, (Fig. 5.2) allowed according to the orbital symmetry theory (Woodward-Hoffmann). For this sigmatropic rearrangement to occur, it is essential that one of the bonds be in the *cis*-configuration. A similar rearrangement is observed for the isomers 9-*cis*,11-*trans* and 8-*trans*,10-*cis* The phenomenon is actually a tool for chemists to produce new isomers. Any given CLA isomer that contains one double bond in the *cis*-configuration and one in the *trans*-configuration can be heated to be isomerized into another specific *cis,trans* or *trans,cis* isomer. Isomers formed might be predicted from formulae as in Fig. 5.2. A simple rule of thumb is that the two double bonds will move against the *cis* end of the bond pairs. For example, 7-*trans*,9-*cis* (a common isomer in milk fat) will isomerize to 8-*cis*,10-*trans* and *vice versa*. Prolonged heating of isomers

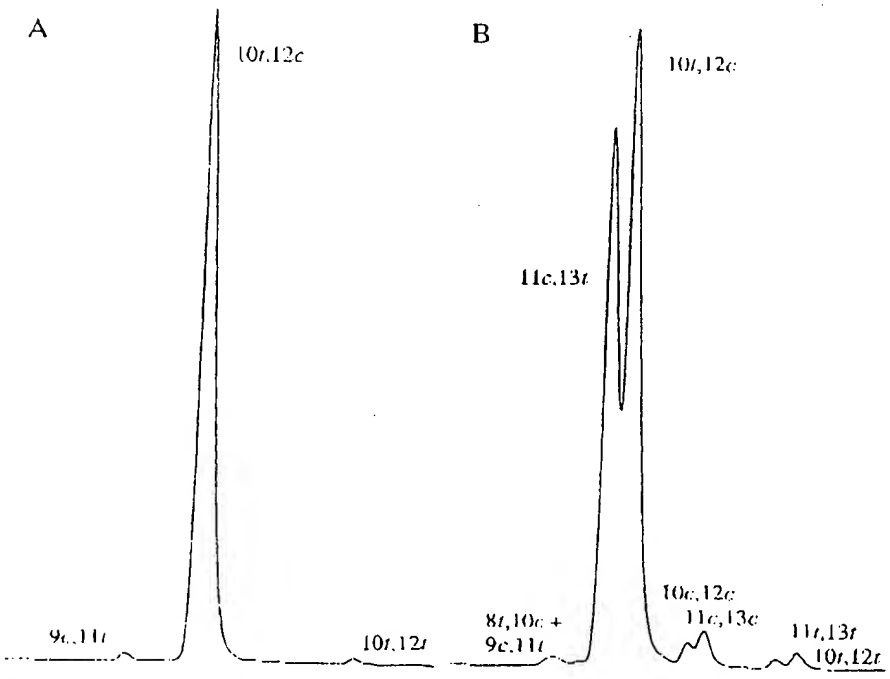
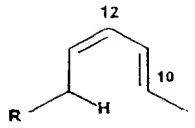


Fig. 5.1. Gas chromatography (GC) profile of ethyl ester of purified 10-*trans*,12-*cis* CLA isomer (a) before and (b) after heating to 220°C in an inert atmosphere for 2 h. The process caused isomerization into the isomer 11-*cis*,13-*trans* by thermal [1,5] sigmatropic hydrogen shift. GC conditions: 100-m CP Sil 88 fused silica capillary column and flame ionization detection (FID).



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Isomer Profile in Avail.

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January-March 2002 by o
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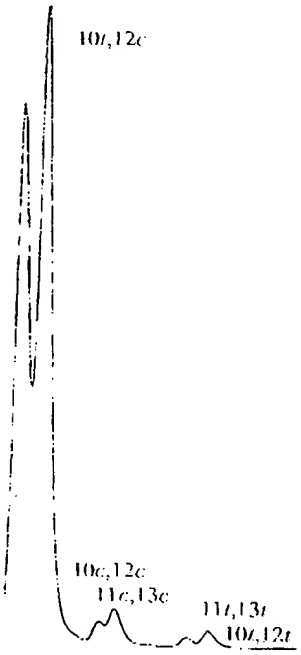

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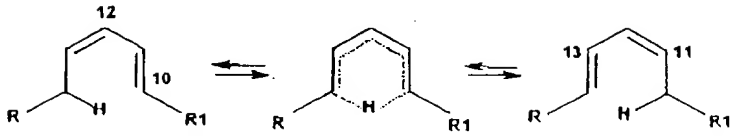

Fig. 5.2. Drawing explaining thermal [1,5] sigmatropic rearrangement between the CLA isomers 10-*trans*,12-*cis* and 11-*cis*,13-*trans*. Reaction is spontaneous and the transition state depicted in the middle is not an intermediate product. R= $-(CH_2)_4$ and R_1 = $-(CH_2)_8CO_2H$.

seems to gradually develop *cis*,*cis* and *trans*,*trans* isomers. Impurities present (iron, copper and other metals) will greatly favor formation of *trans*,*trans* isomers.

Isomer Profile in Available Supplements

The total content of CLA in supplements more or less reflects the starting material. Sunflower oil as a starting material results in ~65% CLA, whereas safflower oil yields up to 80%. Both oils contain a level of palmitic acid that tends to cause precipitation below room temperature. Products are now available with a reduced content of saturated acids and >80% CLA. The products can be classified in two groups, the "4-isomer product" and the "2-isomer product" (Fig. 5.3). The latter product contains almost exclusively 9-*cis*,11-*trans* and 10-*trans*,12-*cis*, both up to ~38% of the oil, or almost 50% of the CLA. The former, however, contains several isomers. The elution order on gas chromatography (GC) of the 4 main peaks is 9-*cis*,11-*trans*; 8-*trans*,10-*cis* (may co-elute with 9-*cis*,11-*trans*);11-*cis*,13-*trans*; and 10-*trans*,12-*cis* (9). In addition a major *trans*,*trans* peak (9,11 and 10,12 co-eluting) often reaches the same level. Such products may contain as little as 8% 10-*trans*,12-*cis*. Despite co-elution, the content of 8-*trans*,10-*cis* can be estimated approximately by measurement of 11-*cis*,13-*trans*. Both are produced to the same degree from their mother components. In other words, the ratio of 11-*cis*,13-*trans* to 11-*cis*,13-*trans* + 10 *trans*,12-*cis* equals that of 8-*trans*,10-*cis* to the co-eluting peak 8-*trans*,10-*cis* + 9-*cis*,11-*trans* (data not published). Products from a single source have been reported to show substantial variation in isomer profile (15), and products also are available that contains virtually no (present data, Table 5.1) or totally lack CLA (10). Two of 17 products sampled and analyzed in January-March 2002 by our laboratory contained high levels of the isomers 11-*cis*,13-*trans* and 8-*trans*,10-*cis* (Table 5.1).

Stability and Breakdown Products of CLA Preparations

Stability of CLA Compared with Linoleic Acid

A few studies report data on the stability of CLA compared with linoleic acid in different test models. Bubbling of oxygen through samples at 90°C resulted in a

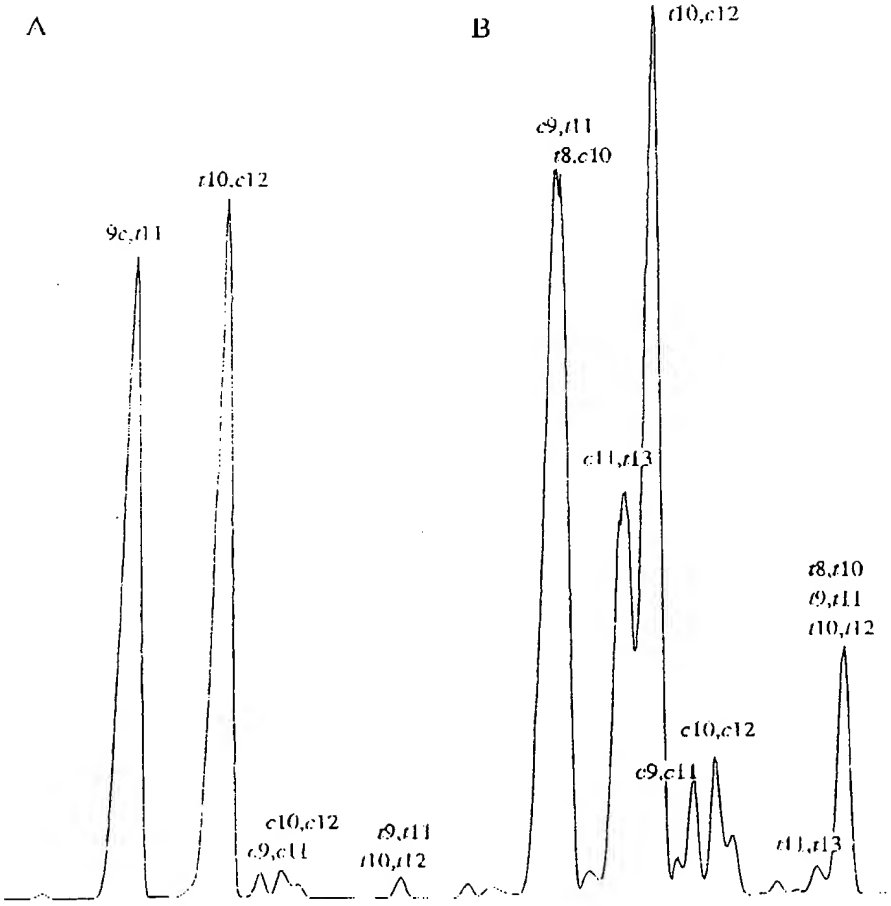

Fig. 5.3. Partial gas chromatography (GC) profile of ethyl esters of (a) a "2 isomer type" and (b) a "4 isomer type" CLA supplement, using a 100-m CP Sil 88 fused silica capillary column and flame ionization detection (FID). Product (a) is identical to product No. 14 and product (b) is identical to No.17 in Table 5.1. Note co-elution of 8-*trans*,10-*cis* and 9 *cis*,11-*trans*.

much higher peroxide value (PV) in linoleic acid (16) than for CLA. When a mixture of CLA isomers was heated to 50°C in air, the rate of oxidation was considerably faster for CLA than for linoleic acid. The rate of oxidation was measured as "remaining CLA" by GC. When comparing groups of CLA isomers, stability decreased in order of *trans,trans* > *cis,trans* or *trans,cis* > *cis,cis*. (17). In a study in aqueous and solvent systems measuring stability by the induction period system,

TABLE 5.1
Content of CLA (% of tot.
January March 2002[a]

Product	Product type
1	Soft gelatine cap
2	Liquid
3	Soft gelatine cap
4	Soft gelatine cap
5	Soft gelatine cap
6	Soft gelatine cap
7	Soft gelatine cap
8	Soft gelatine cap
9	Soft gelatine cap
10	Soft gelatine cap
11	Soft gelatine cap
12	Soft gelatine cap
13	Soft gelatine cap
14	Soft gelatine cap
15	Liquid, emulsion
16	Soft gelatine cap
17	Soft gelatine cap

[a]The isomers 10-*trans*,12-*cis* and
ucts were of the "4 isomer" type
9-*cis*,11-*trans* (not tabulated) due t
trans,12-*cis* in all supplements co
mg KOH/g. (A 100.00% free fatty
region of product 14 and produc

CLA was more stable tha
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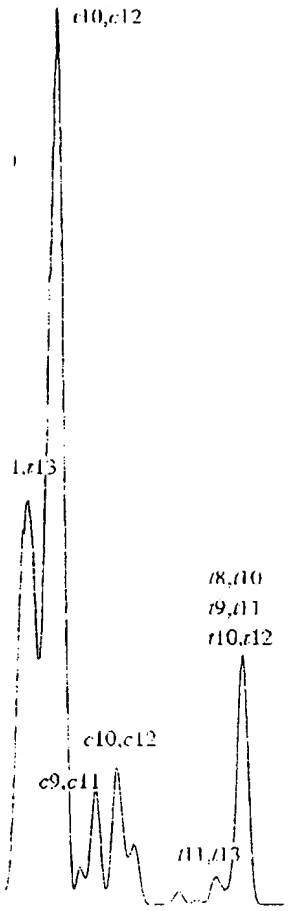

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TABLE 5.1
Content of CLA (% of Total) in 17 Commercial Supplements Sampled in January–March 2002[a]

Product	Product type	Country	%CLA	%10*t*,12*c*	%11*c*,13*t*	Acid value
1	Soft gelatine capsule	Norway	80.1	47.8	0.4	197
2	Liquid	Norway	78.6	47.1	1.8	2
3	Soft gelatine capsule	Norway	69.1	46.7	1.2	196
4	Soft gelatine capsule	Norway	78.3	48.7	0.3	197
5	Soft gelatine capsule	Norway	76.4	46.6	1.3	193
6	Soft gelatine capsule	U.S.	71.4	46.3	0.5	189
7	Soft gelatine capsule	U.S.	74.8	43.1	0.9	192
8	Soft gelatine capsule	U.S.	77.9	48.5	0.3	199
9	Soft gelatine capsule	U.S.	70.8	44.4	0.6	189
10	Soft gelatine capsule	U.S.	79.6	45.3	0.4	193
11	Soft gelatine capsule	U.S.	77.0	44.4	2.3	192
12	Soft gelatine capsule	U.S.	74.3	43.6	1.0	187
13	Soft gelatine capsule	U.S.	61.5	28.5	0.8	180
14	Soft gelatine capsule	U.S.	76.3	48.4	0.3	196
15	Liquid, emulsion	U.S.	1.2	47.8	0.3	NA
16	Soft gelatine capsule	S. Africa	51.7	16.5	16.7	198
17	Soft gelatine capsule	Norway	57.7	29.9	16.5	200

[a]The isomers 10 *trans*,12 *cis* and 11-*cis*,13-*trans* are expressed as the percentage of total CLA. Only two products were of the "4 isomer" type. Two products were liquids, one oil and one emulsion (1.7% fat). Content of 9-*cis*,11-*trans* (not tabulated due to overlap with 8-*trans*,10-*cis*) is approximately equal or slightly less than 10-*trans*,12-*cis* in all supplements currently available. Distilled products typically have acid values of 195–200 mg KOH/g. (A 100.00% free fatty acid product of oleic acid has a theoretical acid value of 198.60). CLA region of product 14 and product 17 is illustrated in Figure 5.3. NA, not available.

CLA was more stable than linoleic acid as free fatty acids, and less stable as ethyl esters (18). Another study using methyl esters reported that stability decreased in the following order: oleate > CLA > linoleate. Samples were stored in the dark at 40°C and monitored by thin-layer chromatography (TLC), GC and PV. From 9-*cis*,11-*trans*, the major monohydroperoxides formed were identified as 8-, 9-, 12- and 13-monohydroperoxides, whereas 10-*trans*,12-*cis* yielded primarily 9-, 10-, 13-, and 14-monohydroperoxides (19).

Data reported on the PV of CLA preparations are consistent with our observations. CLA do not easily develop high PV, yet the oxidative breakdown of CLA seems comparable to that of linoleic acid. The reason is likely to be a more rapid breakdown of peroxides into secondary oxidation products.

Volatiles

In a pilot project on developing a procedure for CLA production, a high content of hexane was observed in a product by headspace GC-mass spectrometry. After searching for the source of contamination, it was finally concluded that pentane

and hexane are among the secondary oxidation products of CLA. This was later confirmed by experiments. To our knowledge, hexane has never been reported to be an important inherent oxidation product of vegetable oils. In a free fatty acid concentrate of 9-*cis*,11-*trans* stored in the dark with air access for 1 wk, the two major volatiles that developed were, not surprisingly, heptanal and 2-nonenal. The concentration increased from 4.8 and 0.7 to 84.6 and 22.5 μg/g, respectively. Volatile breakdown products seem not to build up in soft gelatine capsule supplements. A CLA product that was stored for 5 y at room temperature contained 2.3 μg/g hexanal and 2.2 μg/g heptanal (data not published). No antioxidant was added to the supplement.

Among less volatile breakdown products, furan fatty acids were reported when air was bubbled through CLA dissolved in a mixture of methanol and water at 50°C. (20). Furanoid fatty acids might also arise in preparation of fatty acid methyl esters (FAME) for GC. To our knowledge, furan fatty acids have not been reported as an oxidative breakdown product in dry oil preparations of CLA.

Polymers

Conjugated oils are considered valuable raw materials for the paint and varnish industry because of their film forming properties ("drying") upon air access. This property gives rise to concern regarding the stability of CLA preparations. In a stability test program, 10 mL of CLA triacylglycerols and free fatty acids were stored in an amber open glass bottle in darkness. After 4 mo at 25°C, controls without antioxidants added were highly viscous and not suitable for further stability testing. The samples had a membrane layer on the surface, and the viscosity clearly developed over time. Samples with antioxidants did show a retarded viscosity development (data not published).

Soft gelatine capsules are considered to give reasonable protection from exposure of unsaturated oils to air. Capsules containing CLA free fatty acids showed a slight increase in polymer content from 1% in freshly prepared capsules to 7% after 5 y (data not published). For comparison of health risks, a limit for rejection on cooking oils has been established in some countries; values listed in a report from the European Parliament are 16% (Holland), and 10% (Belgium and Czech Republic) (21).

Stability of CLA in Soft Gelatine Capsules

No data have yet been published on the stability of CLA in capsules. Observations on polymers and volatiles in capsules are reported above. In a stability test program according to International Conference on Harmonization (ICH) guidelines on a free fatty acid product, the content of total CLA was not significantly reduced after 24 mo at 25°C/60% relative humidity. In this test, CLA was measured by GC. Peroxide value (PV) did not develop in the capsules (data not published).

Next Generation Pr

Isomer Purification

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Summary

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for further stability testing.
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ization (ICH) guidelines on
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CLA was measured by GC.
a not published).

Next Generation Products

Isomer Purification

All CLA supplements currently offered contain approximately equal amounts of 9-*cis*,11-*trans* and 10-*trans*,12-*cis*. The extra costs of producing a biased isomer product might be justified if beneficial health effects were documented. The 9-*cis*,11-*trans* and the 10-*trans*,12-*cis* isomers of CLA are now available for research purposes in kilogram scale with a purity of ~90%. In small quantities, purities up to 99% are offered. High yields and high purity can be obtained by repeated crystallization of the methyl ester forms in acetone at temperatures as low as –60°C (22).

A concentrate with 83% 9-*cis*,11-*trans* isomer was obtained from gentle dehydration of ricinoleic acid from castor bean oil and subsequent purification steps (4). The use of urea inclusion compounds does not seem to be a feasible procedure to separate 9-*cis*,11-*trans* and 10-*trans*,12-*cis* (23). Enzymes, however, are promising tools for these separations. A 98% concentrate of 9-*cis*,11-*trans* was reported by using lipase from *Geotrichum candidum*. The enzyme was capable of esterifying selectively 9-*cis*,11-*trans* to monohydric alcohols from a mixture of several isomers (24). A patent has been issued on purification and characterization of isomerases from *Propionibacterium acnes* and *Clostridium sporogenes*. The purified isomerase preparations were able to quantitatively isomerize linoleic acid into the 10-*trans*,12-*cis* isomer of CLA (25).

Triacylglycerols for Food Applications

Free fatty acids and monoalkyl esters are applicable to supplement capsules and probably also to animal feed formulations. However, as an ingredient in food for human consumption, CLA is most attractive as a triacylglycerol. A nonspecific lipase has been reported to esterify CLA with glycerol very efficiently (26). Incorporation of CLA into food fats and oils has also been reported for fish oils (27), butterfat (28,29), and corn oil (30). A bottled triacylglycerol product, stabilized with antioxidants, has been available in the health food market in Scandinavia since 2000. Flavor and antioxidants are added to the oil designed to be taken by spoon. Further technical developments of CLA products improving the stability and applicability as well as addressing specific issues of food legislation will require attention before CLA can be made available as an ingredient for animal feed and human food.

Summary

CLA supplements for human consumption have been available since 1995, and most of the products contain between 60 and 80% CLA in the form of free fatty acids. The history of CLA produced for technical purposes dates back almost 100

y, however. The isomer profile of the supplements range from an almost pure 9-*cis*,11-*trans* + 10-*trans*,12-*cis*-50/50 mixture (made in alcohol solvents between 100 and 150°C), to a mixture with four prominent *cis,trans* or *trans,cis* isomers produced in high alkaline water at high temperatures, of which 8-*trans*,10-*cis* and 11-*cis*,13-*trans* 18:2 are produced from 9-*cis*,11-*trans* and 10-*trans*,12-*cis*, respectively, by thermal [1,5] sigmatropic rearrangements of the isomers. Supplements are typically offered as free fatty acids in soft gelatine capsules. Unpublished data on stability of CLA in capsules stored according to ICH guidelines for 2 y did not show any loss of active ingredient.

Acknowledgments

Per Christian Sæbø and his staff at the laboratory of Natural ASA is acknowledged for patient experimental work on CLA production and purification process developments for the last 5 years. Thanks to Prof. emeritus Lars Skattebøl for valuable comments on migration of sigma bonds.

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APPENDIX D

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Conjugated Linoleic Acid Modulates Tissue Levels of Chemical Mediators and Immunoglobulins in Rats

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ABSTRACT: The effects of conjugated linoleic acid (CLA) on the levels of chemical mediators in peritoneal exudate cells, spleen and lung, and the concentration of immunoglobulins in mesenteric lymph node and splenic lymphocytes and in serum were examined in rats. After feeding diets containing either 0 (control), 0.5 or 1.0% CLA for 3 wk, there was a trend toward a reduction in the release of leukotriene B_4 (LTB_4) from the exudate cells in response to the dietary CLA levels. However, CLA did not appear to affect the release of histamine. A similar dose-response pattern also was observed in splenic LTB_4, lung LTC_4 and serum prostaglandin E_2 levels, and the differences in these indices between the control and 1.0% CLA groups were all statistically significant. The reduction by CLA of the proportions of n-6 polyunsaturated fatty acids in peritoneal exudate cells and splenic lymphocyte total lipids seems to be responsible at least in part for the reduced eicosanoid levels. Splenic levels of immunoglobulin A (IgA), IgG, and IgM increased while those of IgE decreased significantly in animals fed the 1.0% CLA diet. This was reflected in the serum levels of immunoglobulins. The levels of IgA, IgG, and IgM in mesenteric lymph node lymphocytes increased in a dose-dependent manner, while IgE was reduced in those fed the higher CLA intake. However, no differences were seen in the proportion of T-lymphocyte subsets of mesenteric lymph node. These results support the view that CLA mitigates the food-induced allergic reaction.
Lipids 33, 521–527 (1998).

Conjugated linoleic acid (conjugated derivatives of linoleic acid, CLA) exerts diverse physiological effects most of which are favorable to human health. A range of studies has shown a marked alleviating effect of CLA on mammary carcinogenesis (1–4). The mechanism underlying this effect is not yet well understood (5), but continued intake of CLA is not necessarily required for suppression of carcinogenesis (6,7). When considering the diverse effects of CLA, it is reasonable that eicosanoids are involved in the mechanism. The influence of CLA on the metabolic processes leading from linoleic acid to arachidonic acid and, hence, eicosanoids appears to be related to their desirable effects, since CLA tended to reduce the tissue level of prostaglandin E_2 (PGE_2), a putative candidate for a cancer-promoting effect of dietary n-6 polyunsaturated fatty acids (PUFA) (8). In addition, there is a possibility that CLA itself serves as substrate of enzymatic systems for eicosanoid production, as it is shown to undergo desaturation and elongation similar to linoleic acid (9), although it is unknown whether these metabolites could be converted to eicosanoids.

Since the food allergic reaction can readily be modified by the type of dietary PUFA, either n-6 or n-3 (10,11), it is interesting to know if CLA could modify it. The clinical symptom of food allergy is induced by the production of chemical mediators such as histamine and leukotriene (LT) and PG triggered by allergen-specific immunoglobulin (Ig)E (12,13). Our previous studies showed a reduction by CLA of the serum PGE_2 level (8), which is one of the typical chemical mediators in the allergic reaction (12,13). In this context, Belury and Kempa-Steczko (14) showed that CLA reduces the proportion of linoleic acid dose-dependently in hepatic phospholipid and suggested this may result in modified arachidonate-derived eicosanoid production by extrahepatic tissues. More recently, Wong *et al.* (15) reported that CLA modulates certain aspects of the immune defense such as lymphocyte proliferation in mice, although the effect was not always reproduced possibly because of the dependence on the duration of the feeding period. In the present study, we measured the production of chemical mediators and the level of Ig in rats fed different levels of CLA, either 0.5 or 1.0%.

MATERIALS AND METHODS

Preparation of CLA. CLA was prepared according to the method described by Ip *et al.* (16). In brief, 50 g of linoleic acid, purity >99% (Sigma Chemical Co., St. Louis, MO) was dissolved in 290 g of ethylene glycol containing 15 g of NaOH and heated at 180°C for 2 h under nitrogen. After cooling to room temperature, the content was adjusted to pH 4 and extracted with *n*-hexane. The hexane layer was washed with 5% NaCl, dehydrated with 3-A molecular sieves (Nacalai

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Abbreviations: CLA, conjugated linoleic acid; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; LT, leukotriene; MLN, mesenteric lymph node; PEC, peritoneal exudate cells; PG, prostaglandin; POD, peroxidase; PUFA, polyunsaturated fatty acid.

Tesqu, Kyoto, Japan) and dried in a rotary evaporator under nitrogen. The purity of CLA was measured by gas–liquid chromatography (Shimadzu GC-17A, Kyoto, Japan) using a Supelcowax 10 column (0.32 mm × 60 m, film thickness, 0.25 μm; Supelco Inc., Bellefonte, PA). Column temperature was raised from 150 to 220°C at a rate of 4°C/min. The identification of peaks was carried out by the equivalent chain length method (17) and gas chromatography–mass spectrometry (Jeol Auto MS 50, Tokyo, Japan). The purity of CLA preparation was 80.7% with the following composition in percentage: 9*c*,11*t*/9*t*,11*c*, 29.8; 10*t*,12*c*, 29.6; 9*c*,11*c*, 1.3; 10*c*,12*c*, 1.4; 9*t*,11*t*/10*t*,12*t*, 18.6; linoleic acid, 5.6; and others, 13.7.

Animals and diets. The animal experiment adhered to the Kyushu University guidelines for the care and use of laboratory animals. Male, 4-wk-old Sprague-Dawley rats were obtained from Japan Charles River (Atsugi, Japan) and housed individually in a room with controlled temperature and light (20–23°C and lights on 0800–2000 h). After acclimation for 4 d, rats were divided into three groups of 10 rats which were given free access to the experimental diets. The diets were prepared according to the recommendation of the American Institute of Nutrition (AIN-93G diet) (18). The basal diet contained the following ingredients, in g/100 g diet: cornstarch 39.8; casein, 20.0; dextrinized cornstarch, 13.2; sucrose, 10.0; soybean oil, 7.0; AIN-93 mineral mixture, 3.5; AIN-93 vitamin mixture, 1.0; L-cystine, 0.3; choline bitartrate, 0.25; cellulose, 5.0; *tert*-butylhydroquinone, 0.002; and either linoleic acid, 1.0; linoleic acid (Control) and CLA, 0.5 and 0.5; or CLA, 1.0. Thus, LA or CLA was added at the expense of soybean oil in the AIN-93G diet. The fatty acid composition calculated from the composition of individual oils is given in Table 1. Body weight and food intake were recorded every other day. After 3 wk of feeding, five rats were used for collection of the exudate cells and the remaining five rats for other analyses. Blood was withdrawn from the abdominal aorta under light diethyl ether anesthesia and tissues were immediately excised.

Preparation of peritoneal exudate cells (PEC). The method described by Matsuo *et al.* (19) was adopted for the preparation of PEC. Tyrode buffer, consisting of 137 mM NaCl, 2.7 mM KCl, 0.4 mM $NaH_2PO_4 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$, 12 mM $NaHCO_3$, 1.8 mM $CaCl_2 \cdot 2H_2O$, 5.6 mM D-glucose and 0.1% bovine serum fraction V (Boehringer Mannheim GmbH, Mannheim, Germany), pH 7.4, was injected into the rat peritoneal cavity (6 mL/100 g body weight), and the abdomen was gently massaged for 2 min. Then, the cavity was opened, and the buffer containing PEC was recovered with a plastic pipet. The fluid was centrifuged at 200 × *g* for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in Tyrode buffer.

TABLE 1
Fatty Acid Composition of Dietary Fat[a]

Fatty acid	Group		
	Control	0.5% CLA	1.0% CLA
16:0	9.1	9.0	9.0
18:0	3.2	3.42	3.2
18:1	20.4	20.3	20.1
CLA	—	6.4	12.9
18:2	59.7	53.6	47.4
18:3	7.5	7.5	7.4

[a]Fatty acid composition was calculated from the composition of individual component fats, soybean oil, linoleic acid, and conjugated linoleic acid (CLA).

Measurement of leukotriene B_4 (LTB_4) and histamine. LTB_4 was measured as described elsewhere (20–22). PEC (2×10^6 cells) were suspended in Tyrode buffer containing 5 mM calcium ionophore A23187 (Sigma Chemical Co.). After incubating for 20 min at 37°C, 50 mL of the acetonitrile/methanol mixture (6:5, vol/vol) and 50 ng of PGB_2 (Sigma Chemical Co.), as the internal standard, were added. The mixture was kept at –20°C for 30 min and then centrifuged at 1,000 × *g* for 10 min. The supernatant was filtered through a 4-GV 0.22 μm filter (Millipore Corp., Tokyo, Japan). LTB_4 was measured by reversed-phase high-performance liquid chromatography (HPLC) (SCL-10A; Shimadzu Co., Kyoto, Japan) equipped with an ODS-A column (150 × 6.0 mm, 5 μL particle size; YMC, Kyoto, Japan). A mixture of acetonitrile/methanol/water (30:25:45, by vol) containing 5 mM CH_3COONH_4 and 1 mM disodium EDTA, pH 5.6, was used as a mobile phase with a flow rate of 1.1 mL/min. LTB_4 and PGB_2 were detected by absorbance at 280 nm (SPD-10A; Shimadzu Co.). Quantitation of LTB_4 was achieved by comparing the peak area of LTB_4 with that of PGB_2. Histamine content in the culture supernatant was measured fluorometrically (19,23). The intracellular content of histamine also was measured after disrupting the cells by sonication.

Preparation of spleen and mesenteric lymph node (MLN) lymphocytes. Spleen and MLN were excised immediately after withdrawing blood from the aorta, and the tissues were immersed in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) (24,25). The lymphocytes were rinsed three times with the RPMI 1640 medium and filtered to remove tissue scum. To remove fibroblasts, cell suspension was incubated for 30 min at 37°C. Then, 5 mL of the resulting cell suspension was layered on 4 mL of Lympholyte-Rat (Cedarlane, Hornby, Canada) and centrifuged at 1,500 × *g* for 30 min. The lymphocyte band at the interface was recovered, and the cells were rinsed again. The lymphocytes were cultured in 10% fetal bovine serum (Intergen, Purchase, NY) in RPMI 1640 medium at a cell density of 2.5×10^6 cells/mL with or without 2.5 μg/mL of lipopolysaccharide (Bacto lipopolysaccharide B, *Escherichia coli* 026:B6; Difco Laboratories, Detroit, MI). After incubation at 37°C for 24 and 72 h, the concentrations of IgA, IgG, IgM, and IgE were measured by an enzyme-linked immunosorbent assay (ELISA) (26).

T-cell population analysis. Spleen and MLN lymphocytes were analyzed as $CD4^+$- and $CD8^+$-cells by using fluorescein-labeled mouse anti-CD4 (W3/25, mouse IgG1) or phycoerythrin-labeled mouse anti-CD8 (MRC OX-8, mouse IgG1) (both from Serotec Ltd., Kidlington, Oxford, United King-

dom) (23,25). The stained lymphocytes were fixed with 2% paraformaldehyde and analyzed with the EPICS Profile II flowcytometer (Coulter Electronics Ltd., Luto, United Kingdom).

Measurement of serum and culture supernatant Ig by ELISA. Measurements of total Ig were executed using sandwich ELISA methods (24,25). Goat anti-rat IgE, rabbit anti-rat IgG (Fab')2, goat anti-rat IgM (all from Biosoft, Paris, France), and mouse anti-rat IgA (Zymed Lab, San Francisco, CA) were used to fix respective Ig. These antibodies were diluted 1,000 times with 50 mM carbonate-bicarbonate buffer (pH 9.6), and each well of 96-well plates was treated with 100 µL of the solution for 1 h (2 h for IgA) at 37°C. After blocking with 300 µL of the blocking solution overnight at 4°C, each well was treated with 100 µL of the diluted serum or culture supernatant for 1 h (2 h for IgA) at 37°C. Bound IgA was detected by reacting stepwise with 100 µL of peroxidase (POD)-conjugated rabbit anti-rat IgA (1,000 times dilution; Zymed) at 37°C for 2 h, IgG with 100 µL of POD-conjugated rabbit anti-rat IgG (Fab')2 (2,000 times dilution; Cappel, West Chester, PA), and IgM with 100 µL of POD-conjugated goat anti-rat IgM (1,000 times dilution, Cappel) at 37°C for 1 h. Wells were rinsed three times with Tween-20 in phosphate-buffered saline between each step. After incubation at 37°C for 15 min with 100 µL of 1.5% oxalic acid, absorbance at 415 nm was measured with an MPR-A4i ELISA reader (Tosoh, Tokyo, Japan). The bound IgE was detected by reacting with biotin-conjugated mouse anti-rat IgE (2,000 times dilution; Betyl, Montgomery, TX) followed by POD-conjugated avidin (5,000 times dilution, Zymed Lab) at 37°C for 1 h.

Statistical analysis. Data were analyzed by one-way analysis of variance followed by Duncan's new multiple-range test to identify significant differences (27). Values in the text are means ± SE.

RESULTS

Growth performance and tissue weight. As shown in Table 2, there was no difference in food intake and growth of rats for 3 wk among the groups. Thus, the feed efficiency also was comparable among the groups (mean values 0.41 to 0.42). Among tissues weighed, there was a tendency of increasing liver weight and decreasing perirenal adipose tissue weight by dietary CLA and the difference between the linoleic acid and 1.0% CLA groups was significant.

Release of chemical mediators from PEC. PEC isolated from rats fed linoleic acid or CLA were incubated with or without calcium ionophore A23187, and the concentrations of histamine and LTB_4 were measured in the medium. The content of histamine in the cells also was measured to estimate the cellular histamine contents. As shown in Figure 1, the effect of CLA on the release of histamine in PEC was diverse, and there was no significant difference in any of the parameters measured. However, the amounts of histamine stored in the cells tended to decrease with an increasing dietary level of CLA. There was a trend toward a reduction in

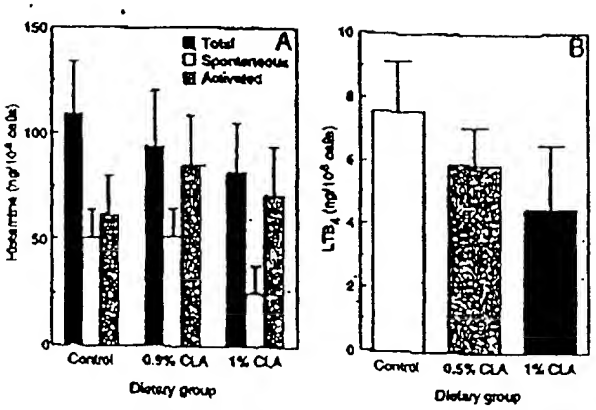

FIG. 1. Effect of dietary conjugated linoleic acid (CLA) on histamine content and release (A) and leukotriene B_4 (LTB_4) release (B) in rat peritoneal exudate cells. Means ± SE of five rats. Histamine release was measured in the presence and absence of calcium ionophore A23187. Total, total amounts of histamine in the cells; Spontaneous, the amount of histamine released during incubation without calcium ionophore A23187; Activated, the amount of histamine which was released from the cells when treated with A23187.

LTB_4 release in response to the dietary level of CLA, but the difference was not significant.

Tissue eicosanoid levels. The effect of CLA on LTB_4 and LTC_4 levels of spleen and lung is shown in Figures 2 and 3, respectively. CLA dose-dependently reduced the level of splenic LTB_4, and the difference between the control and 1% CLA groups was significant. No effect of CLA on the splenic LTC_4 level was observed. However, the concentration of LTC_4 in lung was reduced significantly by CLA even at the 0.5% dietary level. A trend of the dose-dependent reduction of LTB_4 also was observed, but the difference was not significant. The results of the levels of spleen and serum PGE_2 are summarized in Figure 4. CLA significantly reduced the concentration of serum PGE_2, while there was no effect of CLA on the splenic level of PGE_2.

Fatty acid compositions of PEC and splenic lymphocyte lipids. The PUFA composition of PEC and spleen lympho-

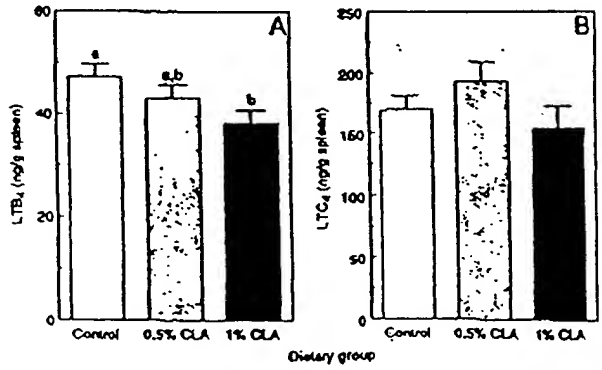

FIG. 2. Effect of dietary CLA on the concentration of splenic (A) LTB_4 and (B) leukotriene C_4 (LTC_4). Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For abbreviations see Figure 1.

TABLE 2
Effects of CLA on Growth and Tissue Weights of Rats[a]

Parameter	Group		
	Control	0.5% CLA	1.0% CLA
Initial body weight (g)	102 ± 1	101 ± 1	102 ± 1
Final body weight (g)	170 ± 2	166 ± 3	162 ± 4
Food intake (g/day)	19.1 ± 0.2	18.9 ± 0.3	18.6 ± 0.3
Tissue weight (g/100 g body weight)			
Liver	4.17 ± 0.09[a]	4.11 ± 0.09[a,b]	4.54 ± 0.07[b]
Kidney	0.85 ± 0.03	0.86 ± 0.03	0.87 ± 0.05
Perirenal adipose tissue	1.41 ± 0.07[a]	1.09 ± 0.09[a,b]	0.97 ± 0.14[b]
Heart	0.40 ± 0.02	0.34 ± 0.04	0.34 ± 0.04
Lung	0.48 ± 0.02	0.52 ± 0.02	0.49 ± 0.01
Spleen	0.22 ± 0.01	0.22 ± 0.01	0.25 ± 0.02
Brain	0.66 ± 0.02	0.70 ± 0.01	0.70 ± 0.01
Testis	0.96 ± 0.04	0.87 ± 0.10	1.00 ± 0.03

[a]Mean ± SE of 5 rats. Control group received 1.0% linoleic acid; 0.5% CLA group, 0.5% each of linoleic and CLA; and 1.0% CLA group, 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at $P < 0.05$. For abbreviation see Table 1.

cyte total lipids is shown in Table 3. There was a dose-dependent reduction by dietary CLA of all n-6 PUFA, 18:2, 20:3, 20:4 and 22:4 in PEC lipids, while there was no difference in the proportion of n-3 PUFA, 22:6 among the groups. A clearer change in these n-6 PUFA was shown in splenic lymphocyte total lipids, and the reduction of 20:4n-6 was significant on a 1.0% CLA diet. Docosahexaenoic acid also tended to decrease with dietary CLA. The decreasing trend of all PUFA in CLA-fed rats was mainly attributable to a moderate increase in major saturated fatty acids, and oleic acid tended to decrease similar to PUFA (data not shown).

Serum thiobarbituric acid value. The concentration of thiobarbituric acid-reactive substance in serum was not modified by dietary CLA, and the values were within 4.1 to 5.5 ng/mL serum in all groups of rats.

Serum Ig levels. As shown in Figure 5, CLA increased the concentration of IgA, IgG and IgM, while decreasing that of IgE in serum. The difference between the control and 1.0% CLA groups was significant in these Ig.

Ig levels in spleen and MLN lymphocytes. Table 4 shows the Ig levels in the medium of rat spleen and MLN lymphocytes cultured for 72 h with or without lipopolysaccharide. Irrespective of the presence or absence of lipopolysaccharide, CLA showed no detectable effects on the Ig levels in spleen lymphocytes except for those of IgM after incubation with lipopolysaccharide, where CLA increased it in a dose-dependent manner. Under the similar situation, CLA increased the concentration of IgA, IgG, and IgM in MLN lymphocytes. The magnitude of the increase was particularly marked at the dietary CLA level of 1.0%. In contrast, there was a significant reduction of the IgE level when the cells from rats fed a 1% CLA diet were incubated with lipopolysaccharide in comparison with the control. A similar response to CLA also was observed even when these cells were incubated for 24 h (data not shown).

Subsets of MLN lymphocytes. The proportion of T-lymphocyte populations of MLN was analyzed as $CD4^+$ and $CD8^+$ subsets. There were no effects of CLA on their relative proportions ($CD4^+/CD8^+$ ratio; 2.6 ± 0.3, 2.4 ± 0.2, and 2.8 ± 0.1 for the control, 0.5% CLA, and 1.0% CLA, respectively).

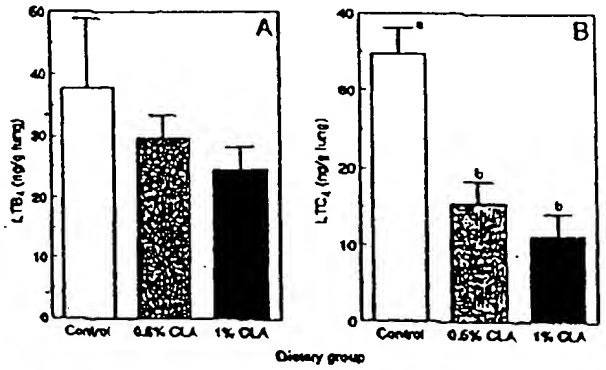

FIG. 3. Effect of dietary CLA on the concentration of lung (A) LTB_4 and (B) LTC_4. Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For abbreviations see Figures 1 and 2.

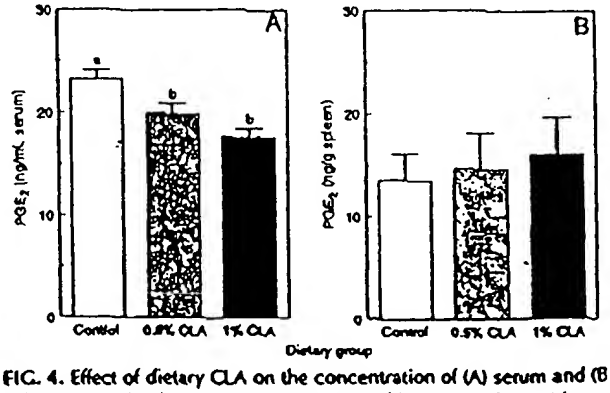

FIG. 4. Effect of dietary CLA on the concentration of (A) serum and (B) spleen prostaglandin E_2 (PGE_2). Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For other abbreviation see Figure 1.

TABLE 3
Effects of CLA on Polyunsaturated Fatty Acid Compositions of Peritoneal Exudate Cells and Spleen Lymphocyte Total Lipids of Rats[a]

Cells and fatty acid	Group		
	Control	0.5% CLA	1.0% CLA
		(wt%)	
Peritoneal exudate cells			
18:2n-6	5.5	5.3	4.2
20:3n-6	0.8	0.7	n.d.
20:4n-6	12.7	11.3	9.0
22:4n-6	5.6	5.3	4.2
22:6n-3	0.6	0.6	0.5
CLA			
9t,11c/9c,11t	n.d.	0.1	0.2
10t,12c	n.d.	0.2	0.2
Spleen lymphocytes			
18:2n-6	12.2 ± 0.8	10.4 ± 0.9	9.3 ± 0.9
20:3n-6	1.6 ± 0.2	1.3 ± 0.3	0.9 ± 0.1
20:4n-6	20.2 ± 0.8[a]	15.4 ± 1.3[a,b]	14.7 ± 1.7[b]
22:4n-6	2.5 ± 0.1	2.0 ± 0.2	1.9 ± 0.2
22:6n-3	1.2 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
CLA			
9t,11c/9c,11t	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
10t,12c	n.d.	0.2 ± 0.0	0.2 ± 0.0

[a]Values are means of two pooled samples from two and three rats each for the exudate cells, and means ± SE of three, five, and five rats for control, 0.5% CLA, and 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at $P < 0.05$; n.d., not detected. For other abbreviation see Table 1.

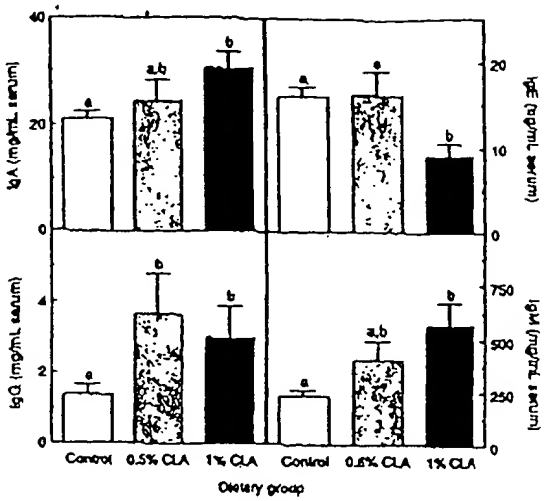

FIG. 5. Effect of dietary CLA on the concentration of serum immunoglobulins (Ig). Mean ± SE of five rats. Values without a common letter (a,b) are significantly different at $P < 0.05$. For abbreviation see Figure 1.

DISCUSSION

The pathway from linoleate to arachidonate and then eicosanoids is crucial to a range of metabolic diseases (28,29). Food allergy is one such disorder, and it is known that some eicosanoids are involved as chemical mediators in the manifestation of clinical symptoms of hypersensitivity (12,13). The inhibitors of LT production have now been clinically adopted (30,31). However, less is known of the effect that food components exert on this process. Although several food components have been shown to reduce eicosanoid production *in vitro*, in most cases it is practically unsatisfactory because of the limited efficacy (21,22). The results of the present study showed that CLA effectively controlled the production of LTB_4, LTC_4, and PGE_2. CLA significantly reduced LTC_4 production in the lung but not in the spleen. A similar tissue-specific reduction of LTC_4 was observed in rats given sesamin and α-tocopherol simultaneously, while in the spleen LTB_4 but not LTC_4 was reduced (21,22). These observations suggest a complex interaction between dietary fat and antioxidants in the LT-producing system.

Numbers of animal studies showed that dietary PUFA effectively modify the production of eicosanoids, and there is an interaction between n-6 and n-3 PUFA (32). PUFA of the n-3 family suppress the production of eicosanoids from arachidonic acid and exert a substantial suppressing effect on carcinogenesis in breast and colon (33,34). However, the anticarcinogenic effect of n-3 PUFA is far less than that of CLA (2–4). Eicosanoid production is known to be dependent on

TABLE 4
Effects of CLA on the Immunoglobulin Production in Splenic and Mesenteric Lymph Node Lymphocytes of Rats[a]

Immunoglobulin	Without lipopolysaccharide			With lipopolysaccharide		
	Control	0.5% CLA	1% CLA	Control	0.5% CLA	1% CLA
Spleen (ng/mL)						
IgA	3.75 ± 1.23	4.83 ± 0.99	3.78 ± 0.96	9.74 ± 2.45	13.6 ± 3.27	8.30 ± 2.50
IgG	51.0 ± 4.6	53.6 ± 2.3	61.5 ± 2.8	68.1 ± 2.4	71.9 ± 1.9	74.4 ± 1.9
IgM	223 ± 22	228 ± 6	246 ± 9	311 ± 9[A]	348 ± 8[B]	394 ± 6[C]
IgE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mesenteric lymph node (ng/mL)						
IgA	1.65 ± 0.13[a]	4.78 ± 1.77[b]	5.05 ± 0.10[b]	2.91 ± 0.23[A]	8.72 ± 0.90[B]	22.3 ± 0.7[C]
IgG	n.d.	3.08 ± 0.69[a]	28.1 ± 4.38[b]	n.d.	4.64 ± 0.11[A]	31.9 ± 4.1[B]
IgM	1.86 ± 0.34[a]	4.74 ± 0.50[a]	96.6 ± 13.4[b]	2.85 ± 0.44[A]	6.36 ± 0.48[B]	122 ± 9[C]
IgE	3.81 ± 0.32	4.02 ± 0.33	3.64 ± 0.47	4.81 ± 0.17[A]	4.52 ± 0.29[A]	3.74 ± 0.21[B]

[a]Means ± SE of five rats. Values without a common superscript letter (A,B,C,a,b,c) are significantly different at $P < 0.05$. The lymphocytes were incubated with or without lipopolysaccharide for 72 h, and the concentration of immunoglobulins (Ig) in the supernatant was measured; n.d., not detected.

the substrate availability (35). CLA reduced the proportion of n-6 PUFA including arachidonic acid in the immune cells as observed in the liver and other tissues (8,14). Because of the limited availability of PEC samples for fatty acid analysis, they were analyzed as two pooled samples from two and three rats each. Though the number of analysis may not permit us to draw a definite conclusion, it seems likely that fatty acid composition of PEC also responded similarly as in spleen lymphocytes. This reduction was at least responsible for the reduced production of LT and PG in these cells. CLA may affect metabolic interconversion of fatty acids in the liver that may ultimately result in modified fatty acid composition and arachidonate-derived eicosanoid production in extrahepatic tissue (14). However, more direct participation of the metabolites of CLA cannot be ruled out (9,36). Therefore, the present study added possible usefulness of CLA for controlling the allergic reaction caused by food. Since the effect of CLA on Ig production differed between MLN lymphocytes and spleen lymphocytes, the analysis of the fatty acid composition of the former cells may provide a clue to understanding the mechanism of action.

In contrast to the eicosanoid production, the level of histamine released from PEC, which reflects the mast cell degranulation by a receptor-independent pathway, apparently was not modified by CLA and more directly the fatty acid composition of membrane phospholipids. Engels *et al.* (37) observed that the type of dietary fats and thus the change in the fatty acid composition of mast cell phospholipids did not influence the cell degranulation process. CLA is reported to be incorporated into triglyceride more preferably than phospholipids in tumor cells (7). Thus, CLA may not substantially influence the fatty acid composition of membrane phospholipids and hence, the structure and function of the membrane. In such a situation, the degranulation of the mast cells may not be modified largely.

An interesting observation is that CLA regulates the Ig production class specifically. Food allergy reaction is initiated by the production of allergen-specific IgE (12,13). IgA, in contrast, serves as an antiallergic factor by interfering with the intestinal absorption of allergen, and IgG also works as an antiallergic factor through the competition with binding of allergen to the receptor on the surface of the target cells such as mast cells and basophiles (12,13). CLA increased the production of IgA and IgG, while reducing that of IgE in lymphocytes, in particular MLN lymphocytes irrespective of the presence or absence of lipopolysaccharide, a cell activator. The response of splenic lymphocytes to CLA was less clear except for a slight but significant increase in IgM after lipopolysaccharide activation. However, the response pattern similar to MLN lymphocytes was observed in serum, indicating that CLA can modify the Ig levels preferably even on a whole-body basis. Bile acids (24) and unsaturated fatty acids (25) also regulate antibody production class specifically, but in a manner contrasted from that of CLA. These compounds may promote the allergic response through an increase in IgE production and a reduction in IgA and IgG production. It is plausible that the production of IgE and of IgA and IgG are at least reciprocally regulated. Thus, in addition to the favorable effect on the eicosanoid production, CLA was expected to mitigate the food allergic reaction.

The amounts of CLA ingested by rats of the present study corresponded to approximately 30 and 60 mg/100 g body weight for 0.5 and 1.0% CLA diets, respectively. These amounts were pharmacological when extrapolated to human, 18 and 36 g/60 kg body weight/day. However, as in the case of weight reduction in man, approximately 3 g/d for 2 to 3 mon, a prolonged ingestion may produce a favorable effect even at a lower dose. A long-term trial with a lower dietary level of CLA merits further study.

In conclusion, CLA produced a situation favorable for mitigation of food allergic reaction. Since the effect was seen at a dietary level as low as 0.5 or 1.0%, it is likely that CLA can strongly regulate multiple metabolic processes. Thus, the clinical application of CLA is warranted. Studies with immunized animals will provide more direct information regarding this issue.

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